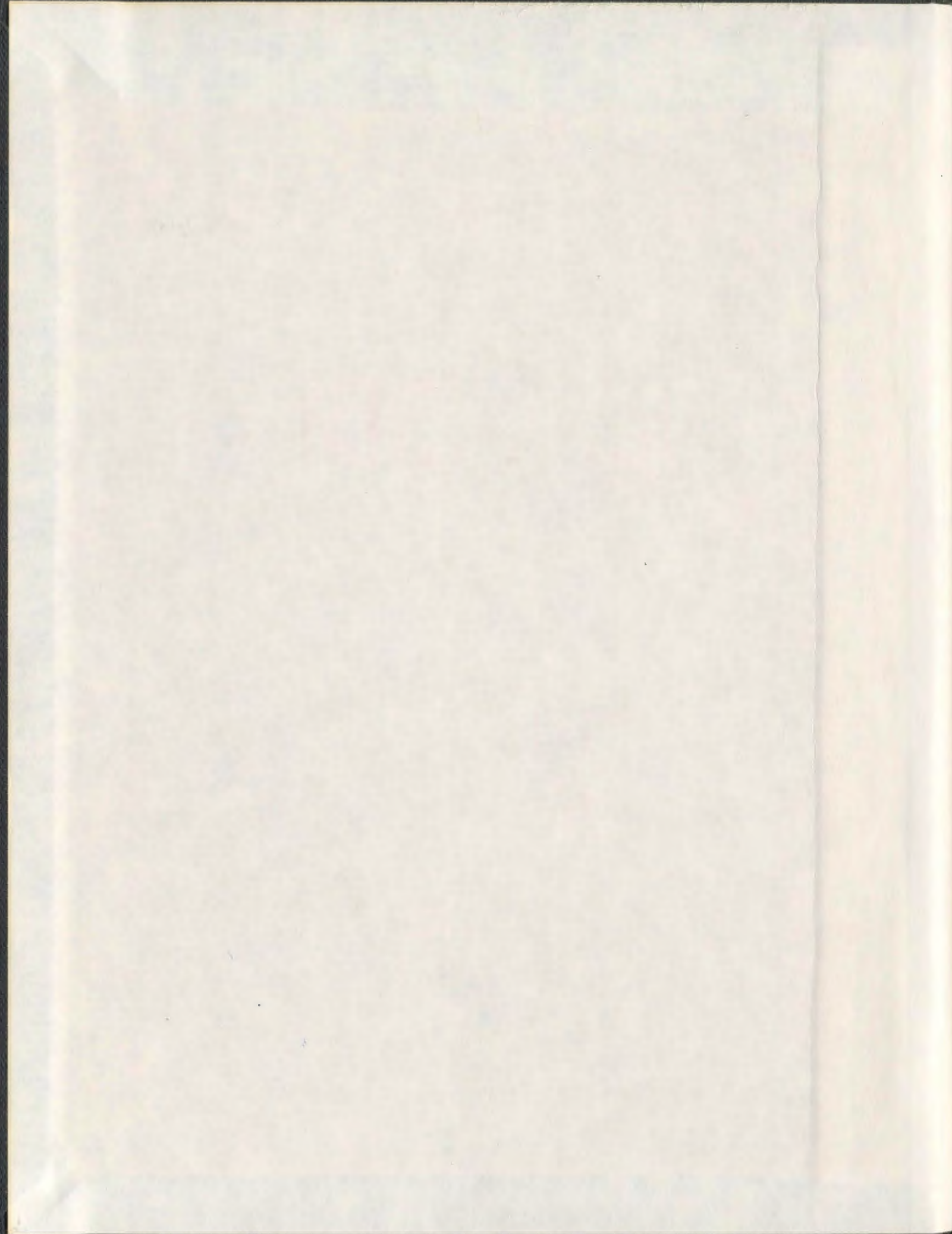


CHARACTERIZATION OF ONCOGENIC HUMAN
PYGOPUS2 EXPRESSION AND REGULATION

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Title page

Characterization of Oncogenic Human Pygopus2 Expression and Regulation

By

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ABSTRACT

Human Pygopus2 (hPYGO2) is a general chromatin remodeler that is overexpressed in, and required for the growth of, a number of tumours of diverse origin and their cell lines. This thesis explores the expression profile of hPygopus2 throughout the cancer cell cycle and identifies novel mechanisms by which it is activated. Examination of hPygopus2 expression and characterization of factors that enhance its expression could lead to better understanding of the signalling networks at play in cancer. It was hypothesized that hPygopus2 expression is induced in the G1 phase of the cell cycle by factors that promote cell cycle progression. The tumour specific expression of hPYGO2 and its requirement for cell proliferation, specifically at the G1/S transition, suggests that hPYGO2 induction is closely correlated with the cell cycle.

In a set of normal and cancer cell lines, hPygopus2 mRNA and protein levels were significantly reduced in G₀, highest in G1 and moderate in S and G2/M relative to unsynchronized samples. This cell cycle dependent expression suggested that hPygopus2 may be a marker of the G1 phase in proliferating cells. Furthermore, hPYGO2 protein levels in the G2/M phase relative to the G1 phase were inversely proportional to cell cycle length, highlighting a potential utility of hPYGO2 in determining tumour cell proliferation rate.

The requirement of hPygopus2 for cancer cell proliferation and its high expression during the G1 phase suggested that it is induced during this phase. Due to its well established role in breast cancer (BrCa), the induction of hPygopus2 by 17 β -estradiol (E₂) was studied. High levels of E₂ and its receptor, Estrogen receptor alpha

(ER α), are well established risk factors for the development and progression of BrCa. However, our knowledge of ER α transcriptional complex components (and their dynamics) at E₂ target gene promoters, particularly in ER α negative (ER α -) BrCa, is incomplete. E₂ treatment of BrCa cells enhanced hPygopus2 mRNA and protein expression via the binding of ER α and SP1 transcription factor (SP1) complexes at the *hPYGO2* promoter. Promoter occupancy of these transcription factors required an intact estrogen response element half-site and GC-box and functional DNA binding domains of both proteins. While ER α binding to the *hPYGO2* promoter could be prevented by pretreatment with the ER α antagonist Fulvestrant, SP1 binding was only reduced by RNAi. The ability of these proteins to independently occupy the *hPYGO2* promoter suggested that SP1 may still play a role in *hPYGO2* activation in ER α - BrCa. In ER α - BrCa cells, SP1 expression was required for the activation of *hPYGO2* and several other “ER α -SP1” target genes and a reduction of SP1 resulted in cell cycle arrest. Together, these findings suggested that in ER α + BrCa, ER α and SP1 have important roles in the regulation of hPygopus2. The ability of SP1 to modulate hPygopus2 expression in the absence of ER α , suggested that hPYGO2 expression might assist in chemotherapy selection in endocrine disruptor unresponsive BrCa tumours.

The previously demonstrated induction of hPygopus2 by the E74-like factor 1 (ELF1), a Retinoblastoma (RB) tumour suppressor regulated protein, suggested that deregulation of the RB-ELF1 axis leads to overexpression of hPygopus2. The role of the human papillomavirus (HPV) in the inactivation of RB is well characterized. Evidence was provided for hPYGO2 as a cellular biomarker expressed in response to gene

deregulation by the main effector of HPV, the E7 oncoprotein, in cervical cancer (CxCa). hPYGO2 levels were greater in high-grade lesions and squamous cell carcinomas as compared to normal epithelia. Similarly, hPygopus2 mRNA and protein levels were greater in HPV-positive CxCa cells relative to uninfected primary cells. RNAi mediated depletion of HPV-E7 increased, while ELF RNAi decreased, association of RB with the *hPYGO2* promoter in CxCa cell lines. Transfection of dominant active RB inhibited ELF1-dependent activation of *hPYGO2*, while ELF1 itself increased hPygopus2 expression. Chromatin immunoprecipitation assays showed that RB repressed hPygopus2 by inhibiting ELF1 at the E26 transformation specific binding site in the *hPYGO2* promoter. These results suggested that abrogation of RB by E7 resulted in derepression of ELF1, which in turn stimulated expression of hPygopus2. Thus, initiation of hPygopus2 expression by ELF1 was required for proliferation of CxCa cells and its expression therefore may act as a surrogate marker for dysplasia.

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LIST OF ABBREVIATIONS

4-OHT.....	4-Hydroxytamoxifen
AF-1.....	Activation function 1 domain
AF-2.....	Activation function 2 domain
AP-1...	Activator protein-1, heterodimeric transcription factor composed of FOS and JUN
APC/APC.....	Adenomatous polyposis coli gene/protein
arm.....	armadillo protein, <i>D. melanogaster</i> homologue of human β -catenin
AXIN/AXIN.....	Axin gene/protein
β -Actin.....	actin, beta protein
β -Catenin.....	catenin (cadherins-associated protein), beta protein
β -gal.....	β -galactosidase
BCL9.....	B-cell CLL/lymphoma 9 protein
bp.....	Base pair
BrCa.....	Breast cancer
CO ₂	Carbon dioxide
CCNA/CCNA.....	Cyclin A gene/protein
CCND/CCND.....	Cyclin D gene/protein
CCNE/CCNE.....	Cyclin E gene/protein
cDMEM.....	Complete DMEM (DMEM+10% FBS)
CDK.....	Cyclin dependent kinase protein
cDNA.....	complimentary DNA
ChIP.....	Chromatin immunoprecipitation
CIN.....	Cervical intraepithelial neoplasia
CIP.....	CDK interacting protein
CSNK1/2.....	Casein kinase I/II protein
CTSD/CTSD.....	Cathepsin D gene/protein
CxCa.....	Cervical cancer
d.....	Day(s)
DAPI.....	4',6-diamidino-2-phenylindole
DBD.....	DNA binding domain
dH ₂ O.....	Deionized water
DMEM.....	Dulbecco's modified eagle's medium
DMSO.....	Dimethyl sulfoxide
DNA.....	Deoxyribonucleic acid
dtFBS.....	dextran treated and charcoal stripped FBS
DVL.....	Dishevelled, dsh homologue 1 (<i>Drosophila</i>) protein
E ₂	17 β -estradiol (estrogen)
E2.....	E2 region of HPV
E7.....	E7 oncoprotein of HPV
E2F.....	E2F transcription factor protein
EBS.....	ETS binding site

EDTA.....	Ethylenediaminetetraacetic acid
ELF1.....	E74-like factor (ets domain transcription factor) protein
ER(α).....	Estrogen receptor (alpha) protein (ESR1 synonym)
ER α -/+.....	Estrogen receptor alpha negative/positive
ERE.....	Estrogen response element
<i>ESR1/ESR1</i>	Estrogen receptor 1 gene/protein
Et.....	Ethanol (95%)
ETS.....	E26 transformation specific
FBS.....	Fetal bovine serum
FITC.....	Fluorescein isothiocyanate
<i>FOS/FOS</i>	FBJ murine osteosarcoma viral oncogene homologue gene/protein
FUL.....	Fulvestrant, Faslodex or ICI 182, 780
FZD.....	Frizzled family receptor protein
G ₀	G zero non-proliferating state (cell cycle phase)
G1.....	Gap 1 (cell cycle phase)
G2.....	Gap 2 (cell cycle phase)
GC-box.....	GC rich promoter binding motif
GSK3B.....	Glycogen synthase kinase 3 beta protein
h.....	Hour(s)
HCCS1.....	Hepatocellular carcinoma suppressor 1 protein
HH3.....	Histone H3 protein (phosphorylated, pHH3)
HRP.....	Horseradish peroxidase
HPV.....	Human papillomavirus
INK4.....	Inhibitor of CDK4 protein
IgG.....	Immunoglobulin G
JUN.....	Jun proto-oncogene protein
kDa.....	Kilodaltons
KIP.....	Kinase inhibitory protein
LBD.....	Ligand binding domain
LEF.....	Lymphoid enhancer binding factor
lgs.....	legless protein, <i>D. melanogaster</i> homologue of human BCL9
LRP.....	Low density lipoprotein receptor related protein
LxCxE.....	Leucine-x-cysteine-x-glutamic acid protein binding motif
μ g.....	Microgram(s)
μ l.....	Microliter(s)
μ M.....	Micromolar
m.....	Minute(s)
M.....	Mitosis
mg.....	Milligram(s)
MKI67.....	Antigen identified by monoclonal antibody Ki-67 protein
ml.....	Milliliter(s)
mM.....	Millimolar
MMTV.....	Mouse mammary tumour virus

mRNA.....	Messenger ribonucleic acid
NFKB.....	Nuclear factor of kappa light polypeptide gene enhancer in B-cells protein
ng.....	Nanogram(s)
nM.....	Nanomolar
NHD.....	N-terminal homology domain
NLS.....	Nuclear localization sequence
p15.....	Cyclin dependent kinase inhibitor 2B protein
p16INK4a.....	Cyclin dependent kinase inhibitor 2A protein
p18.....	Cyclin dependent kinase inhibitor 2C protein
p19.....	Cyclin dependent kinase inhibitor 2D protein
p21.....	Cyclin dependent kinase inhibitor 1A protein
p27.....	Cyclin dependent kinase inhibitor 1B protein
p57.....	Cyclin dependent kinase inhibitor 1C protein
pan.....	pangolin protein, <i>D. melanogaster</i> homologue of human LEF
PBS.....	Phosphate buffered saline
pcDNA3.1.....	pcDNA3.1 empty expression vector
PCNA.....	Proliferating cell nuclear antigen protein
pcER α	ER α expression vector
pcER α -DBM.....	ER α DNA binding domain mutant expression vector
PCR.....	Polymerase chain reaction
pcSP1.....	SP1 expression vector
pcSP1-DBM.....	SP1 DNA binding domain mutant expression vector
PHD.....	Plant homeodomain
pGL3-basic.....	pGL3 luciferase reporter vector
pGL3-48.....	pGL3 containing 48 bases upstream of <i>hPYGO2</i> start site
pGL3-225.....	pGL3 containing 225 bases upstream of <i>hPYGO2</i> start site
pGL3-531.....	pGL3 containing 531 bases upstream of <i>hPYGO2</i> start site
pGL3-531 mutERE.....	pGL3-531 containing a mutated ERE half-site at -331
pGL3-531 mutGC.....	pGL3-531 containing a mutated GC-box at -356
pGL3-531 mutERE+GC.....	pGL3-531 containing mutated ERE half-site and GC-box
pGL3-829.....	pGL3 containing 829 bases upstream of <i>hPYGO2</i> start site
pGL3-1143.....	pGL3 containing 1143 bases upstream of <i>hPYGO2</i> start site
pGL3-1494.....	pGL3 containing 1494 bases upstream of <i>hPYGO2</i> start site
PP2A.....	Protein phosphatase 2A protein
PRF-DMEM.....	Phenol red free DMEM
<i>PYGO</i> / <i>PYGO</i>	Pygopus homologue (<i>Drosophila</i>) gene or mRNA or cDNA/protein
Q-PCR.....	Quantitative/real time polymerase chain reaction
<i>RARA</i> / <i>RARA</i>	Retinoic acid receptor, alpha gene/protein
<i>RBL1</i> / <i>RBL1</i> or p107.....	Retinoblastoma like 1 gene/protein
<i>RBL2</i> / <i>RBL2</i> or p130.....	Retinoblastoma like 2 gene/protein
RNAi.....	RNA interference
S.....	Synthesis (cell cycle phase)
SCC.....	Squamous cell carcinoma
SDS.....	Sodium dodecyl sulfate

siELF1.....	<i>ELF1</i> targeting siRNA
siNTC.....	Non-targeting control siRNA
siPy2-X.....	<i>hPYGO2</i> exonic region targeting siRNA
siPy2-Z.....	<i>hPYGO2</i> intronic region targeting siRNA
siRNA.....	Small interfering RNA
SP1.....	SP1 transcription factor protein
TBS-T.....	Tris buffered saline with tween 20
TCF	Transcription factor (T-cell factor) protein
TFDP2.....	Transcription factor Dp-2 (E2F dimerization partner 2) protein
<i>TGFA</i> / <i>TGFA</i>	Transforming growth factor, alpha gene/protein
<i>TNF</i> / <i>TNF</i>	Tumor necrosis factor gene/protein
TMX.....	Tamoxifen, Nolvadex or ICI 46, 474
wg.....	Wingless protein, <i>D. melanogaster</i> homologue of human WNT
WNT.....	Wingless-type MMTV integration site family member protein

CO-AUTHORSHIP STATEMENTS

Chapter 2:

Figures and tables in this chapter were presented, in modified form, as a part of a poster presentation at the 100th Annual American Association for Cancer Research Conference (Tzenov and Kao, 2008). Experiments were performed by Y. Tzenov. Dr. Kao assisted with the experimental design and analysis.

Chapter 3:

Figures and tables in this chapter were presented, in modified form, as a part of a poster presentation at the 103rd Annual American Association for Cancer Research Conference (Tzenov, Andrews and Kao, 2013). Experiments were performed by Y. Tzenov. P. Andrews and Dr. Kao assisted with the experimental design and analysis.

Chapter 4:

This research has been published in Molecular Cancer Research (Tzenov et al., 2013). Additionally, some figures and tables in this chapter were presented, in modified form, as a part of poster and oral presentations at the 27th International Papillomavirus Conference (Tzenov, Popadiuk and Kao, 2011). Experiments were designed, performed and analysed with the assistance of Dr. K. Kao.

Chapter 1: Molecular Basics of Cancer

1.1 Foreword

“While there are several chronic diseases more destructive to life than cancer, none is more feared.”

— Charles Mayo

Charles H. Mayo and William A. Hendricks, 'Carcinoma of the Right Segment of the Colon', presented to Southern Surgical Assoc. (15 Dec 1925).

In *Annals of Surgery* (Mar 1926), **83**, 357.

1.2 Physiological and pathological types of tissue growth

Cells in proliferating tissues are generated and maintained by a small pool of self-renewing and rarely dividing stem cells (Weinberg, 2007d). During tissue growth and repair, stem cells divide asymmetrically to produce one new stem cell (to maintain the population) and one rapidly dividing transit amplifying cell. The transit amplifying cells divide until the tissue has grown to the appropriate size or the damage has been repaired. At this time they switch from proliferation mode to differentiation mode (lose proliferation capacity) and mature to form the properly functioning tissue.

Tissue growth can be induced by mitogens under either physiological or pathological circumstances (Robbins, 2007a). If the cell morphology remains indistinguishable from normal cells and if removal of the stimulus that evoked the change causes cells to return to their normal size/number, then the process is regarded as physiological. Two examples of cell stimulation, which always remain subject to normal

regulatory mechanisms, include hypertrophy (increase in cell size) and hyperplasia (increase in cell number).

Once cell proliferation becomes uncoordinated from physiological regulation it becomes disproportionate and results in neoplasia (Robbins, 2007b). Neoplasia refers to a range of progressive conditions which begins with the slight delay in cell differentiation, due to the abnormal proliferation of undifferentiated stem cells and transit amplifying cells, and eventually ends with the complete inability of cells to differentiate.

This multistage process is driven by the gradual accumulation of genetic mutations, epigenetic alterations and/or increased mitogenic stimulation, all of which increase the activity of proteins that induce cell proliferation (oncogenes), and decrease the activity of proteins that restrict it (tumour suppressor genes) (Weinberg, 2007a; Weinberg, 2007f). The stem cells (evolving units), which acquire these genetic alterations, gain proliferation advantages and begin to divide more frequently thereby generating more transit amplifying cells which quickly outgrow their neighbouring normal cell counterparts (Weinberg, 2007d).

The abnormal expansion of immature stem cells (and the corresponding decrease in mature cells) within an epithelium is the earliest form of a precancerous lesion and is referred to as dysplasia (or intraepithelial neoplasia) (Robbins, 2007b). Dysplasia can be subdivided into low grade, in which the risk of transformation is low, and high grade, which is a more advanced progression toward malignancy.

Abnormally proliferating stem cells in this population will eventually experience additional mutations, which makes their proliferation even more efficient and rapid and,

therefore, increases the likelihood of future genetic hits (Weinberg, 2007d). With the accumulation of each mutation, stem cells produce a different clone that will eventually dominate the local tissue environment and is responsible for genetic heterogeneity in tumours.

Carcinoma *in situ* is a localized premalignant condition in which cells cease to differentiate all together, resulting in very rapid cell proliferation and the complete loss of tissue identity (Robbins, 2007b). Eventually, the rate of genetic change outpaces the rate at which natural selection and the elimination of less fit subclones can occur (Weinberg, 2007d). While the accumulation of these mutations may not affect the growth of the current neoplasm, it may be beneficial during later stages (metastasis and chemotherapy resistance) by adding variability to the cell population.

When a tumour reaches a certain volume and its cells invade through the basement membrane and metastasize, it is referred to as invasive malignant carcinoma (Weinberg, 2007c). As insidious as primary tumours are, metastatic dissemination of tumour cells is responsible for 90% of morbidity and mortality in cancer.

Metastasis is characterized by a complex process called "the invasion-metastasis cascade" (Weinberg, 2007c). After breaching the basement membrane, cells can intravasate into either lymphatic or blood microvessels, which permits their transport to distant anatomical sites. At these sites, the cells become trapped and subsequently extravasate into organs to form micrometastases. Eventually these micrometastases acquire the ability to colonize the tissue in which they reside and form macrometastases.

The final stage of tumour progression and behaviour is its response to treatment. Treatment of disseminated cancer consists of a combination of surgery to remove visible tumours and systemic chemotherapy to kill cancer cells within micrometastases (Page and Takimoto, 2002). Chemotherapeutic agents take advantage of the principle that rapidly dividing cells will be more susceptible at a given dosage. Thus, these antineoplastic drugs are meant to eliminate the majority of rapidly dividing tumour cells, but in doing so, they avoid the infrequently dividing cancer stem cells (Reya et al., 2001). Unfortunately, rapidly dividing normal cells such as hair follicles, gastrointestinal epithelial cells and immune cells are susceptible to chemotherapy and thus, may cause adverse side effects (Page and Takimoto, 2002).

1.3 Molecular and cellular regulation of cell proliferation

1.3.1 Physical and spatial restrictions

As all cells are arranged in tight three dimensional networks (tissues or neoplasms), their proliferation requires the disruption of intercellular and cell-substratum interactions (Makrilia et al., 2009). The four major groups of cell adhesion molecules include cadherins, integrins, selectins, and immunoglobulin superfamily members. Once these adhesion molecules are down regulated and cells attain the capacity to divide, they must be stimulated in order to progress through the cell cycle.

1.3.2 Regulatory components involved in cell proliferation

Cell replication includes the duplication of chromosomes (during the synthesis, S, phase) and other cellular components (during the gap, G1 and G2, phases) in the parental cell and their distribution (during mitosis or M phase) to the daughter cells, which are formed by physical separation (cytokinesis) (Morgan, 2007b).

A complex network of regulatory proteins governs progression through the cell cycle phases (Morgan, 2007a). In particular, progression from G1 into S is regarded as the most important transition. During G1 the cell compiles and processes a wide array of extracellular and intracellular signals and decides, at the restriction point in late G1, whether or not it will complete another entire cell cycle. Passing the restriction point represents an irreversible commitment to cell cycle entry, after which the cell becomes unresponsive to extracellular signals and the remaining cell cycle events and phase transitions occur in a cell autonomous manner.

The G1/S transition, and other transitions, are very specific and highly regulated events overseen by the cell cycle control system (Morgan, 2007c). The cyclin-dependent kinases (CDKs), a family of serine-threonine kinases, are the central components of this system. Like other kinases, these very stable enzymes catalyze the attachment of phosphate groups to protein substrates. CDK activity, which oscillates throughout the cell cycle, leads to the cyclic activation and inhibition of regulatory proteins (via phosphorylation) that initiate specific cell cycle phase events.

The phosphorylation-mediated regulation of CDK targets is achieved by phase-specific CDKs (CDK1 for M, CDK2 for G1/S and S, and CDK4 and 6 for G1), which are

primarily governed by their interaction with the cyclin proteins (Morgan, 2007c). As the levels of the different cyclin proteins dramatically change throughout the cell cycle, their phase-specific interaction with CDKs leads to oscillations in CDK activity. Therefore, cyclins are also categorized according to cell cycle phase [Cyclin D (CCND) is high in G1, Cyclin E (CCNE) is high during G1/S, Cyclin A (CCNA) is high during S and Cyclin B is high during M]. So, each cyclin-CDK complex is active during a specific phase and thus responsible for the initiation of the critical events within that phase (Figure 1.1).

An additional level of regulation includes CDK inhibitors (CKIs), which can bind to cyclin-CDK complexes (or CDKs alone) and inactivate them (Morgan, 2007c). CKIs are separated into two groups based on their function. The CDK interacting protein (CIP)/kinase inhibitory protein (KIP) family, which includes Cyclin dependent kinase inhibitors 1A (p21), 1B (p27) and 1C (p57), is responsible for promoting cell cycle arrest in response to unfavorable environmental or intracellular conditions. The more specifically targeting group of CKIs belongs to the inhibitors of CDK4 (INK4) family and includes Cyclin dependent kinase inhibitor 2A (p16INK4a), 2B (p15), 2C (p18) and 2D (p19). These proteins promote cell cycle arrest when cells encounter anti-proliferative signals. The involvement of CKIs in cell cycle progression is usually uncommon and reserved for abnormal situations (such as cancer). Typically, cell cycle progression, especially through the G1/S transition, is primarily dependent on the levels of cyclins as CDK levels remain fairly constant throughout the cell cycle.

The principal factors responsible for transcribing cyclins belong to the E2F transcription factor (E2F) family (Morgan, 2007c). E2F complexes are heterodimers

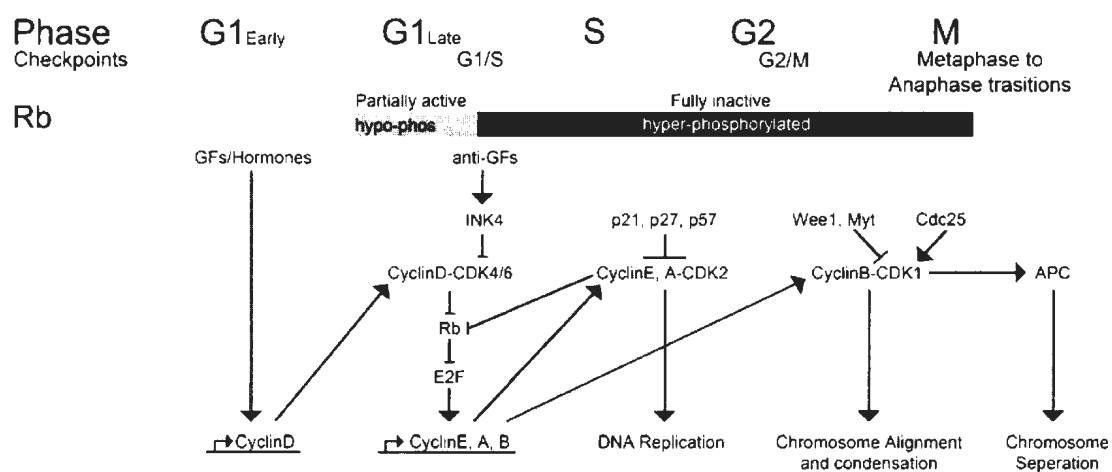
composed of one E2F subunit and one transcription factor Dp-2 (E2F dimerization partner 2) (TFDP) subunit. While these complexes preside over the expression of thousands of genes, their most critical targets are *CCNE* and *CCNA*.

The transcriptional activity of E2F-TFDP complexes is regulated by the retinoblastoma family of transcriptional repressors (Nevins, 1998; Dyson, 1998). The Retinoblastoma (RB or RB1) protein, the prototype of this family, and its related pocket proteins Retinoblastoma like 1 (RBL1 or p107) and 2 (RBL2 or p130) each interact with specific E2F-TFDP complexes to inhibit gene expression (Masciullo et al., 2000).

RB directed inhibition of E2F factors is achieved by two separate but complementary means (Morgan, 2007c). First, the RB proteins recruit histone modifying enzymes (such as histone deacetylases) and chromatin remodeling complexes (such as Switch/Sucrose non-fermentable), which modify the local formation of chromatin such that it is unfavorable for transcription. Second, the RB proteins bind to the transcriptional activation domain of E2F factors and thereby block their activity.

RB-mediated inhibition of E2F results in the down regulation of genes specifically required for the G1/S transition (Weinberg, 1995). This enables RB to provide a braking mechanism for proliferating cells and to permit cell cycle exit. It is important to mention that E2F complexes are not the only targets of RB. RB has been shown to bind to and inhibit the activity of several transcription factors (Morris and Dyson, 2001), thereby preventing transcription of several other genes required for cell cycle progression, notably the E74-like factor 1 (ELF1) (Wang et al., 1993).

Figure 1.1 Overview of the regulatory components involved in cell cycle progression and their main functions (generated from information in Morgan, 2007c).



As RB is a nuclear phosphoprotein, its ability to inhibit gene expression is dependent on its phosphorylation status (Harbour and Dean, 2000). RB is in an active state when it is un- or hypophosphorylated and hyperphosphorylation leads to a conformational change, which prevents RB from binding to target protein such as E2F and ELF1. In order for cells to progress through the G1/S transition, RB must be inactivated, and remain inactivated, so that the cell cycle progression genes can be properly expressed and cell cycle progression can occur.

1.3.3 Cell signalling induces progression through the restriction point

In multicellular organisms cell division and all other cell activities and actions (growth, differentiation, migration, apoptosis) are governed by a complex communication system known as cell signalling (Alberts et al., 2008). Entry into the cell cycle occurs only when a cell is exposed to the appropriate extracellular mitogens (Morgan, 2007a). These hormones, amino acids or peptides are attached to or secreted from neighboring cells or present within the extracellular matrix.

An extensive array of receptors, present on or within receiving cells, interpret/mediate these growth factors (Alberts et al., 2008). The two largest classes of receptors include the cell surface bound receptors and the nuclear receptors. Cell surface receptors can be further subdivided into G-protein coupled receptors, enzyme coupled receptors and proteolysis associated receptors/mediators. Ligand binding to any of these receptors initiates one or more intracellular signalling cascade(s) through several downstream adaptors, enzymes and signalling cascade components, which finally lead to

the activation of specific transcription factors. Signalling via nuclear receptors is more direct. The ligand migrates through the cell and nuclear membranes and binds to its appropriate receptor, which then becomes an activated transcription factor.

The activation of one or multiple transcription factors leads to induction of large cohorts of genes and activation of genetic programs (Weinberg, 2007b). While these pathways can lead to multiple outputs (proliferation, growth, differentiation, migration, and/or apoptosis), that are dependent on ligand type, cell type (proteome) and stage of development: at one point or another they all have the capacity to activate transcription of *CCND*. Under most conditions, expression of Cyclin D is the first and perhaps most important prerequisite for cell cycle progression. Mitogen induced expression of *CCND* occurs early in the G1 phase of the cell cycle because this is the only time that cells are sensitive to extracellular cues. Expression of *CCND* leads to formation of *CCND*-CDK4/6 complexes which phosphorylate the RB protein and changes its phosphorylation status from unphosphorylated to hypophosphorylated (Sherr, 1996). The moderately lowered activity of RB as a result of this phosphorylation causes a proportion of the E2F-bound RB to dissociate. The resulting activation of E2F leads to transcription of *CCNE* and *CCNA* and translation of their gene products, Cyclin E and Cyclin A, respectively. These proteins form complexes with CDK2 to hyperphosphorylate and fully inactivate RB (Lundberg and Weinberg, 1998; Harbour et al., 1999). As inactivation of RB is the only prerequisite for cells to progress through the restriction point, cancer cells have found several ways to achieve this.

1.3.4 Cell signalling, gene mutations and cancer

Both normal and cancer cells utilize common mechanisms to promote proliferative growth, but in cancer cells relatively minor, yet critical modifications occur to remove normally existing restrictions on growth and/or unconditionally stimulate growth signalling (Weinberg, 2007b).

The highly organized architecture of tissues acts as a potent tumour suppressor, thus, the disruption of cell adhesion and breakdown of tissue integrity is a prerequisite for abnormal cell proliferation (Crossin, 1991). Deregulation of cell interactions provides cells with the physical capacity to divide. This eventually leads to anchorage independent growth and overcomes contact inhibition.

Once cells are capable of cell division, they are induced to proliferate by either acquiring genetic mutations or receiving excessive mitogenic stimulation (Weinberg, 2007b). Both these events activate/increase levels or inactivate/decrease levels of cell cycle control system components (cyclins, CDKs, CKIs, RB) and/or internal cell signalling components (ligands, receptors, adaptors and enzymes, signalling cascade components and/or transcription factors) in a manner which allows the cell to gain a proliferative advantage.

1.3.5 Translation of intracellular signalling into cancer diagnosis and therapy

While several cell cycle components are deregulated in a multitude of cancers, only some have been shown to be useful as diagnostic biomarkers and even fewer have been utilized as targets. For example, the p16INK4a tumour suppressor is part of a

feedback loop between RB and CDK4/6 and is up regulated when RB is phosphorylated for extended periods (Gupta et al., 2010). It is overexpressed in cervical neoplasia as a result of functional inactivation of RB by the human papillomavirus (HPV) E7 oncoprotein. As its expression correlates with degree of cervical squamous and glandular dysplasia, p16INK4a has become a biomarker for diagnosis in addition to its prognostic and predictive utility.

The cell signalling components which have been utilized as therapeutic targets contain active sites that can be bound (and thereby blocked) by either small molecule drugs or monoclonal antibodies (the two main types of rationally designed targeted therapies) (Weinberg, 2007e). While monoclonal antibody targets are restricted to the cell surface (because of their size), small molecule drugs are able to diffuse through the plasma membrane and target intracellular proteins.

A prominent example of a successful small molecule drug is Tamoxifen (TMX, Nolvadex or ICI 46, 474), the first targeted therapy. TMX interferes with 17β -estradiol (estrogen, E_2) binding to the Estrogen receptor ($ER\alpha$) (Ali et al., 2011) and thereby prevents the transcription of target genes required for the proliferation of breast cancer (BrCa) cells. Unfortunately the success of TMX, and other $ER\alpha$ antagonists, notably fulvestrant (FUL, Faslodex, ICI 182,780), is not common place, especially in the potentially more relevant Wnt signalling pathway.

The Wnt signal transduction cascade is one of the most powerful pathways in the cell. During development, it is involved in critical processes like body axis patterning and tissue morphogenesis (Hatsell et al., 2003; Reya and Clevers, 2005). In adults, it regulates

homeostasis in a myriad of tissues including breast, intestine and blood, and maintains stem cell populations. Thus, as expected, acquired mutations in genes encoding components of the Wnt cascade are implicated in the initiation and progression of several cancers, including breast, colorectal and cervical (Polakis, 2007; Clevers, 2006). Wnt signalling is particularly important because of its role in stimulating cancer stem cells, the driving force behind many (if not all) cancers.

Unfortunately, the limited number of pathway components that are amenable to small molecule inhibition has hampered development of targeted Wnt pathway inhibitors (Ding et al., 2007). Dickkopf 1, a Wnt receptor antagonist which inhibited differentiation of human osteoblast precursor cells that lead to multiple myeloma (Qiang et al., 2008) and pyrvinium, a selective inhibitor of casein kinase I (CSNK1), which also promotes degradation of several Wnt signalling pathway components [such as β -Catenin, AXIN and human Pygopus (PYGO) 2] (Thorne et al., 2010) are some notable successes.

1.4 Wnt signalling and Pygopus

1.4.1 Wnt signalling

Wnt signalling is initiated when the Wingless type MMTV integration site family member (WNT; wingless, wg, in *Drosophila*) ligands, secreted from neighboring cells, bind to Frizzled (FZD)/low density lipoprotein receptor-related protein (LRP) cell surface complexes on target cells (Logan and Nusse, 2004) (Figure 1.2). Through Disheveled (DVL), this receptor complex transduces an inhibitory signal to the β -Catenin (armadillo,

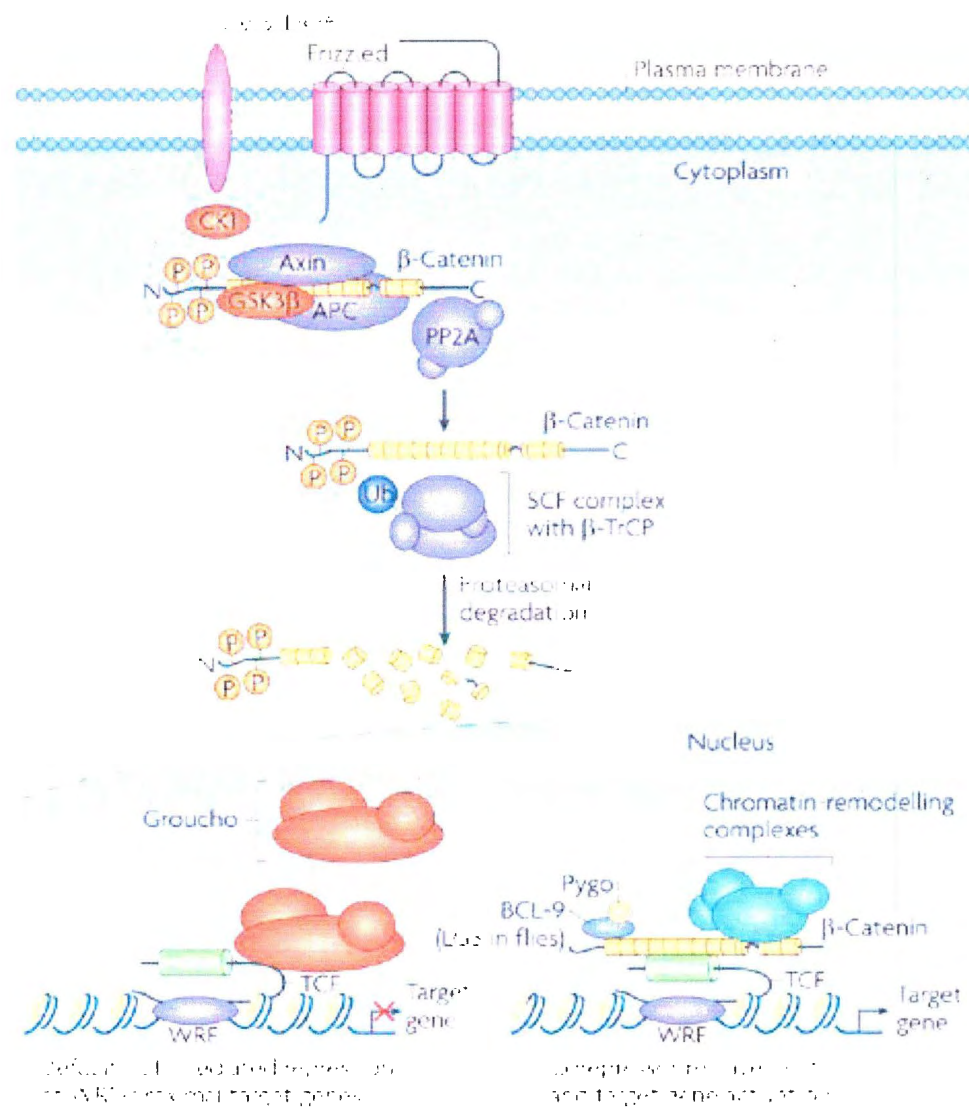
arm in *Drosophila*) destruction complex, which consists of AXIN, Adenomatous polyposis coli (APC), Glycogen synthase kinase 3 beta (GSK3B), Casein kinase 1 (CSNK1) and Protein phosphatase 2A (PP2A) (Clevers, 2006; Klaus and Birchmeier, 2008). When active, this complex is responsible for the continuous degradation of β -Catenin and thereby the maintenance of its low cytoplasmic levels. Inhibition of the destruction complex permits the cytoplasmic accumulation and eventually nuclear translocation of β -Catenin. In the nucleus, β -Catenin interacts with the Lymphoid enhancer binding factor (LEF; pangolin, pan, in *Drosophila*)/Transcription factor (T-cell factor) (TCF) proteins and with the recruitment of transcriptional coactivators, such as B-cell CLL/lymphoma 9 (BCL9; legless, lgs, in *Drosophila*) and PYGO, Wnt target gene transcription commences.

Although the first Wnt gene was discovered three decades ago (Nusse and Varmus, 1982), additional components of this pathway and other interacting factors are still being discovered.

1.4.2 Pygopus

In 2002, three independent laboratories identified *Drosophila pygopus* as a component of the Wnt signalling pathway (Belenkaya et al., 2002; Parker et al., 2002; Thompson et al., 2002). The same year, a fourth laboratory identified legless as a binding partner of pygopus (the interaction between which is mediated by the evolutionarily conserved plant homeodomain, PHD, of pygopus) that exerts its function by physically linking pygopus to armadillo (Kramps et al., 2002). Subsequently Pygopus was shown

Figure 1.2 Schematic diagram showing the different components of the β -Catenin dependent Wnt signal transduction pathway (adapted from Mosimann et al., 2009).



not only to anchor armadillo/ β -Catenin in the nucleus (Townesley et al., 2004; Tolwinski and Wieschaus, 2004; Krieghoff et al., 2006) but also to exhibit essential transcriptional coactivator activity (Hoffmans et al., 2005; Stadel and Basler, 2005; Mosimann et al., 2006). This activity is mediated by the N-terminal Homology Domain (NHD, which contains a nuclear localization sequence and is unique to Pygopus proteins) of pygopus or by the factors that it recruits. The observation that inhibition of pygopus function produces embryonic and adult phenotypes consistent with loss of wingless signalling established pygopus as a dedicated nuclear core component of the Wnt/wg pathway (Belenkaya et al., 2002; Parker et al., 2002; Thompson et al., 2002; Kramps et al., 2002). In higher organisms, the Wnt dedicated function of Pygopus is not as straight forward.

Mammals and amphibians have two Pygopus homologues, *PYGO1* and *PYGO2*, unlike flies, which have one gene (Belenkaya et al., 2002; Kramps et al., 2002; Thompson et al., 2002; Lake and Kao, 2003; Li et al., 2004). Because deletion of both genes produced the same developmental defects as deletion of *PYGO2* alone, the function of *PYGO2* appeared to be dominant (Schwab et al., 2007). However, the ablation of Pygopus genes in mice did not phenocopy mutants with loss of Wnt signalling as it did in flies (Song et al., 2007; Schwab et al., 2007; Li et al., 2007). The results yielded by these, and other studies, support the role of Pygopus in some but not all Wnt-mediated processes in mammals.

The Wnt-dependent processes in which PYGO is involved include lung morphogenesis (Li et al., 2007), kidney (Schwab et al., 2007), pancreas (Jonckheere et al., 2008) and mammary gland (Gu et al., 2012) development, adult brain patterning

(Lake and Kao, 2003) and body axis formation (Kennedy et al., 2010). The Wnt-independent roles of Pygopus include its requirement for eye development (Song et al., 2007), spermiogenesis (Nair et al., 2008) and embryonic brain patterning (Lake and Kao, 2003).

While there is no single clear explanation as to why human and *Drosophila* Pygopus proteins have different degrees of dedication to Wnt/wg signalling, there are some results which are consistent with the evolution of human Pygopus into a general chromatin remodeler and transcriptional activator.

Due to a single amino acid difference between the *Drosophila* and human PHD domain of Pygopus, only human PYGO can bind methylated lysine residues on histone tails (Fiedler et al., 2008; Kessler et al., 2009; Miller et al., 2010). Furthermore, this interaction can occur both in the presence and absence of BCL9 (a Wnt transcription coactivator). These results are consistent with a role of hPYGO2 that is less confined to Wnt signalling.

The broader role of Pygopus in chromatin regulation and as a general transcriptional coactivator was verified by the finding that its NHD can interact with histone modifying enzymes [CREB binding protein (Andrews et al., 2009), myeloid/lymphoid or mixed lineage leukemia 2 (Chen et al., 2010)], chromatin remodeling complexes [SPT-ADA-GCN5 acetylase complex (Chen et al., 2010)], transcriptional coactivators [mediator complex subunit 12 and 13 (Carrera et al., 2008)] and general transcriptional machinery [TAF4 RNA polymerase II, TATA box binding protein-associated factor (Wright and Tjian, 2009)]. The essential role of Pygopus in

several processes and the need to identify novel cancer biomarkers, led to studies examining its role and requirement for cancer.

1.4.3 The expression, requirement and function of human Pygopus2 in cancer

The essential role of human Pygopus2 (hPYGO2) in several types of cancers is well established with studies initially carried out in our laboratory. Our group showed that hPYGO2 was overexpressed in epithelial ovarian cancer cell lines and patient samples and that its knockdown halted growth in the cell lines (Popadiuk et al., 2006). *hPYGO2* was also up regulated in several BrCa lines and malignant breast tumours and was required for the proliferation of BrCa cells (Andrews et al., 2007). hPYGO2 protein overexpression in gliomas exhibited a positive correlation (Wang et al., 2010), and was significantly associated (Chen et al., 2011), with tumour grade. It was also required for proliferation of glioma (Wang et al., 2010) and glioblastoma cells (Chen et al., 2011), specifically arresting cells in the G1 phase when knocked down. In contrast, overexpression of hPYGO2 in glioma cells promoted proliferation by enhancing progression through the G1/S checkpoint (Chen et al., 2011). Brembeck et al. expanded our knowledge of hPYGO2 in the colon by showing that its expression increased in adenomas and colon tumours compared to normal tissues (Brembeck et al., 2011). Lastly, Moghbeli et al. showed that *hPYGO2* mRNA was overexpressed in esophageal squamous cell carcinoma (Moghbeli et al., 2013). Thus, while the up regulation and requirement of hPygopus2 in cancer has been frequently observed, understanding the functional role of hPYGO2 in cancer remains a challenge.

Knockdown of Pygopus in colorectal cancer cells reduced Wnt reporter activity (Thompson et al., 2002) suggesting a Wnt signalling modulatory role in these cells. The association of hPYGO2 with histone modifying enzymes (myeloid/lymphoid or mixed lineage leukemia 2 and SPT-ADA-GCN5 acetylase complex) augmented Wnt-dependent transcription and led to the expansion of BrCa stem-like cells (Chen et al., 2010). In HeLa (cervical adenocarcinoma) cells, stable overexpression of hPYGO2 exhibited anti-apoptotic properties by counteracting vinblastine induced cell death (De et al., 2009). Recent data from our lab (Andrews et al., Accepted) suggests that the interaction between hPYGO2 and the nucleolar protein Treacher Collins Franceschetti syndrome 1 was required for transcription of ribosomal DNA in a variety of cancer cell lines. Another recent study demonstrated that acute deletion of *hPYGO2* in MMTV-Wnt1 transgenic mice decreased tumor initiation capability and that chronic loss of hPYGO2 delayed mammary tumour development (Watanabe et al., 2013).

These observations raise the possibility that Pygopus has multiple functions in cancer, but despite its requirement and overexpression, few studies have examined what factors are responsible for hPygopus2 up regulation.

1.4.4 The regulation of hPygopus2 by E74-like factor 1

In a previous study from our lab (Andrews et al., 2008), ELF1 was found to associate with the *hPYGO2* promoter and modulate its activity. ELF1 knockdown resulted in a decrease in *hPYGO2* promoter activity and overexpression of ELF1 caused an increase in endogenous *hPYGO2* mRNA levels.

ELF1 belongs to the E26 transformation specific (ETS) family of transcription factors (Thompson et al., 1992). The 27 ETS members are identified by the highly conserved 85 amino acid ETS DNA binding domain (DBD), which recognizes the core GGAA/T sequence (ETS binding site, EBS) (Seth and Watson, 2005). The EBS is found in numerous promoters, consistent with the ability of ETS factors to act as positive or negative gene regulators. ELF1 specifically was initially described as a lymphoid specific protein because it bound to several lymphoid gene promoters (Thompson et al., 1992), but has subsequently been shown to have a role in a wide variety of processes such as development (Janknecht et al., 1989; Jin et al., 2009; Calero-Nieto et al., 2010; Choi et al., 2011), mitogenesis (Moreau-Gachelin et al., 1988), oncogenesis (Moreau-Gachelin et al., 1988; Seth et al., 1989) and viral gene activation (Markovitz et al., 1992; Leiden et al., 1992; Clark et al., 1993). As deregulation of some of these processes leads to carcinogenesis, the role of ETS factors, including ELF1, in cancer cannot be understated.

Several studies examining the expression of ELF1 in cancer have been conducted. ELF1 is overexpressed in osteosarcoma (Bassuk et al., 1998), BrCa (Scott et al., 2000), prostate cancer (Gavrilov et al., 2001), endometrial carcinoma (Takai et al., 2003b), cervical cancer (Nicol et al., 2008) and glioma (Sahin et al., 2009). Furthermore, its expression in several types of cancer correlates with other well-established biomarkers.

ELF1 expression in non-small cell lung cancer correlated with Survivin (Yang et al., 2010) and Vascular endothelial growth factor (Yang et al., 2009) protein expression and was associated with metastasis, differentiation, clinical stage and prognosis. ELF1 expression is also correlated with the Proliferating cell nuclear antigen (PCNA) protein

labelling index, clinical stage, histological grade, invasion and clinical outcome in endometrial carcinomas (Takai et al., 2004) and epithelial ovarian carcinoma (Takai et al., 2003a). As these correlations are quite noteworthy and significant, it is surprising that there have only been three studies in which ELF1 function has been examined.

As mentioned earlier, ELF1 activated *hPYGO2* in BrCa (Andrews et al., 2008). ELF1 also bound to the promoters of the Hepatocellular carcinoma suppressor 1 (*HCCSI*) gene in liver cancer (Zhu et al., 2006) and to the TEK tyrosine kinase, endothelial gene in melanoma (Huang et al., 2006), and induced their expression.

The expression, subcellular localization, and protein-protein interactions of ELF1, all of which affect its ability to bind promoters, is dependent upon post-translational modifications. For example, O-GlcNAc glycosylation and phosphorylation (Juang et al., 2002) of ELF1 increases its molecular weight from 68 kilodaltons (kDa) to 98 kDa, but also causes its translocation to the nucleus where it binds to target gene promoters. While promoter bound ELF1 is active, transcription is prevented due to its interaction with the RB tumour suppressor protein (Wang et al., 1993). Specifically, the pocket region of RB binds to the LxCxE motif within the transactivation domain of ELF1 and thereby RB inhibits the transcriptional activity of ELF1. Interestingly, it was determined that only the active (hypophosphorylated) form of RB was able to bind ELF1.

The RB protein plays a critical role in cell cycle regulation and as a result exhibits cell cycle dependent activity. Due to its association with RB, it has been suggested that the transcriptional activity of ELF1 would also be cell cycle dependent. As ELF1 appears

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to be able to activate *hPYGO2* gene expression in cancer, it is possible that hPYGO2 expression might be linked to the cell cycle.

1.5 Goals of thesis

1.5.1 How is human Pygopus2 expressed during cell cycle progression?

Hypothesis: If human Pygopus2 is induced by E74-like factor 1, then it will be expressed in a cell cycle dependent manner.

Objectives: (1) Obtain cells in each phase of the cell cycle.
(2) Measure human Pygopus2 mRNA and protein levels at each cell cycle phase.

1.5.2: How does 17 β -estradiol affect human Pygopus2 expression in breast cancer?

Hypothesis: If human Pygopus2 expression is fundamentally linked to cell cycle, then 17 β -estradiol will induces it via Estrogen receptor alpha.

Objectives: (1) Treat cells with 17 β -estradiol and determine if hPygopus2 expression increases.
(2) Determine if Estrogen receptor alpha is required for 17 β -estradiol induction of human Pygopus2.

1.5.3 How does infection of cervical cancer cells by human papillomavirus E7 induce human Pygopus2 expression?

Hypothesis: If human Pygopus2 is regulated by Retinoblastoma and E74-like factor 1, then deregulated E7 expression will result in human Pygopus2 overexpression.

Objectives: (1) Determine if Retinoblastoma and E74-like factor 1 can modulate human Pygopus2 expression.
(2) Determine if E7 modulation affects human Pygopus2 expression.

Chapter 2: Cell cycle dependent expression of hPygopus2

2.1 Introduction

The Wnt/ β -Catenin transcription component, Pygopus, was originally identified as a protein required for Wnt dependent transcriptional activation during embryonic development (Jessen et al., 2008), but is also overexpressed in, and required for the growth of, a number of cancer cell lines of diverse origin (Popadiuk et al., 2006; Andrews et al., 2007; Wang et al., 2010; Chen et al., 2011; Moghbeli et al., 2013). Because of its specific utilization by malignant cells, hPygopus2 may serve as a cancer-specific biomarker of diagnostic, prognostic and/or predictive benefit. However, a detailed characterization of its expression in cancer cells, which would likely be tied to its regulation, is lacking.

An important factor required for hPYGO2 expression in breast and cervical carcinoma cell lines is the ELF1 protein (Andrews et al., 2008), an ETS family member. ETS factors have well-established roles in carcinogenesis, regulating oncogenes and tumour suppressors involved in apoptosis, angiogenesis, invasion and metastasis (Seth and Watson, 2005). ELF1 itself is overexpressed in several cancers (Bassuk et al., 1998; Scott et al., 2000; Gavrillov et al., 2001; Nicol et al., 2008; Sahin et al., 2009) in which its expression correlates with poor prognosis (Takai et al., 2003b; Yang et al., 2009; Yang et al., 2010) and directly activates genes involved in tumourigenesis (Seth and Watson, 2005). In normal resting T lymphocytes, the pocket region of the Retinoblastoma tumour suppressor interacts with the N-terminal LxCxE motif of promoter bound ELF1 and blocks its transactivation (Wang et al., 1993).

The predominant function of RB is in the cell cycle, where it provides a braking mechanism that regulates transition from G1 to S. Cell cycle progression requires the inactivation of RB in a cell cycle phase specific manner (Lundberg and Weinberg, 1998). In G₀ and during early G1, RB is in its active hypophosphorylated state and interacts with promoter bound transcription factors, such as ELF1, to inhibit their activity. Mitogen stimulation in late G1 activates cyclin-CDK complexes which hyperphosphorylate and inactivate RB, causing it to release and allow activation of transcription factors. The cell cycle phase specific regulation of ELF1 by RB suggests that ELF1 activity and its ability to transcribe target genes is cell cycle dependent. Thus, the regulation of *hPYGO2* by ELF1 establishes a direct link between *hPYGO2* and the cell cycle.

I undertook a general correlative study to examine if the pattern of expression of *hPygopus2* varied with different phases of the cell cycle. In this chapter, I will provide evidence that both *hPygopus2* mRNA and protein exhibit cell cycle dependent expression in MCF7 BrCa cells. My data additionally indicated that the cell cycle dependent expression of *hPygopus2* is a general phenomenon and is observed in several normal and cancer cell lines. I further show that relative protein levels of *hPYGO2* between different cell cycle phases are inversely proportional to cell cycle length. These data suggest the potential for *hPYGO2* to be developed as a proliferation marker to estimate tumour cell growth rate.

2.2 Materials and Methods

2.2.1 Cell line maintenance

HeLa (cervical adenocarcinoma), HEK293 (human embryonic kidney), MCF7 (ER α + breast adenocarcinoma), MDA-MB-231 (ER α - breast adenocarcinoma), SKOV-3 (ovarian adenocarcinoma), LNCaP (androgen sensitive prostate carcinoma) and PC-3 (androgen insensitive prostate adenocarcinoma) cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen) at 37°C with 5% carbon dioxide (CO₂).

2.2.2 Cell collection

Cells were washed once with 1X Phosphate Buffered Saline (PBS) (Sigma) and treated with trypsin (Invitrogen) for 2-4 minutes (m) at 37°C. Trypsin was inactivated by the addition of DMEM+10% FBS (referred to as complete media, cDMEM) and suspended cells were transferred to 1.7 milliliter (ml) Eppendorf tubes. Cells were pelleted by centrifugation at a g-force of 4600 for 2 m and then washed again with PBS. Cells were pelleted by centrifugation and the PBS was aspirated.

2.2.3 Serum deprivation and release

HeLa, HEK293 and MDA-MB-231 cells were seeded at a density of 1.5×10^5 cells/well while MCF7, PC-3, SKOV-3 and LNCaP cells were seeded at a density of 3×10^5 cells/well in six well plates and grown for 1 day (d) in cDMEM. An unsynchronized

cell sample was collected at the 1 d time point. The remainder of the cells were washed once with PBS and then incubated in Phenol Red-Free DMEM (PRF-DMEM) without FBS for 4 d. A G₀ phase cell sample was collected at this time. The remainder of the cells were washed once with PBS, incubated in cDMEM and collected at various time points. HeLa, HEK293 and MDA-MB-231 cells were collected at 6 and 12 hours (h) time points and then every 3 h for 57 h. MCF7, PC-3, SKOV-3 and LNCaP cells were collected at 6, 12 and 18 h time points and then every 3 h for 3 d. Two samples were collected at each time point and used for flow cytometry cell cycle analysis and protein extraction for immunoblot analysis.

It is important to note that after the addition of serum supplemented media, cells undergo a recovery period (Pollack et al., 1990). This is a time during which cells recover from the stress associated with serum deprivation and prepare to enter the cell cycle. The length of this period is cell type specific (approximately 12-25 h) but is generally longer for slower growing cells and shorter for faster growing cells.

2.2.4 Double thymidine block

The double thymidine block was used to arrest cells in the S phase. MCF7 cells were seeded at a density of 3×10^5 cells/well in six well plates and grown for 1 d in cDMEM. After this period, cells were washed once with PBS and incubated in cDMEM containing 10 millimolar (mM) thymidine for 21 h. The cells were washed once with PBS and cultured for an additional 8 h in cDMEM. Subsequently, cells were washed with PBS and incubated cDMEM containing 10 mM thymidine for 16 h. After this period cells were collected as described earlier (Section 2.2.2).

2.2.5 Paclitaxel treatment

Paclitaxel (taxol) treatment was used to arrest cells in the M phase. MCF7 cells were seeded at a density of 3×10^5 cells/well in six well plates for 1 d. Cells were washed once with PBS and incubated in cDMEM containing 2 μ M paclitaxel for 2 d. Cells were collected as described earlier (Section 2.2.2).

2.2.6 Flow cytometry

Collected and pelleted cells (Section 2.2.2) were fixed by re-suspension with PBS containing 2% formaldehyde and incubated for 10 m at 37°C. Fixed cells were placed on ice for 1 m. Then, cells were pelleted and permeabilized by re-suspended in 90% methanol/1X PBS and incubation for 30 m on ice). Subsequently cells were pelleted, washed once with PBS and re-suspended in 300-600 microliters (μ l) of PBS. One microlitre of ten milligrams (mg)/ml propidium iodide and 20 μ l of 10 mg/ml RNase were added to the cell suspensions, which were then incubated in the dark for 20 m at 37°C.

A minimum of 10, 000 cells from each sample were analyzed by flow cytometry using a BD FACS Calibur bench top flow cytometer and the Cell Quest Pro operating program. The percentage of cells in each phase (for each sample) was calculated and graphically displayed (distribution plots) using ModFit analysis software.

It is important to note that propidium iodide is a fluorescent dye which binds to DNA in a linear fashion (no sequence preference). Thus, because this method is based on DNA quantity, it cannot distinguish between G₀ and G₁ and between G₂ and M phases.

However, the G_0 denoted cells are serum deprived, a well-established method for arresting cells in this phase (Pollack et al., 1990). Additionally, both G2 and M phases are very short relative to other phases (G1 and S) so mRNA and protein levels should not change considerably between these two phases.

2.2.7 Cell cycle length determination

The cell cycle phase distribution for each sample was plotted on a line graph. The X-axis was time (that sample was collected) and the y-axis was percentage of cells. There was a line for each phase ($G_0/G1$, S and G2/M). The time between the two S phase peaks was considered as the approximate cell cycle length.

2.2.8 RNA extraction and cDNA generation

Total cellular RNA was isolated from pelleted cells (Section 2.2.2) using the nucleospin RNA II kit (Macherey-Nagel) as per the manufacturer's instructions. cDNA was generated by reverse transcribing 1 microgram (μg) of total RNA using 5X First strand buffer, 100 nanograms (ng)/ μl of random hexamers, 100 mM Dithiothreitol, 100 mM deoxtribonucleotides, MgCl_2 , Moloney murine leukemia virus reverse transcriptase and RNA Guard as per Invitrogen's protocol.

2.2.9 Quantitative PCR

Two micromicroliters of newly synthesized cDNA (Section 2.2.8) was used for the quantitative real-time PCR (Q-PCR) reaction. cDNA samples were analyzed by real-time reverse transcription PCR (ABI Prism 7000 detection System) using SYBR green

(Applied Biosystems). Dissociation (melting) curves confirmed a single PCR amplicon in all reactions. Results were analyzed using the comparative Ct method ($2^{-\Delta\Delta C_t}$) (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). This included normalization of the genes of interest to the control genes followed by normalization of experimental samples to the control samples (ie. *hPYGO2* normalized to *β -Actin*, then the unsynchronized sample was set to 1 and the dH₂O, dimethyl sulfoxide (DMSO), G₀, G1, S and G2/M samples were adjusted accordingly). Primers are listed in appendix Table 6.1.

2.2.10 Protein extraction, SDS-PAGE and immunoblotting

Protein was extracted from pelleted cells (Section 2.2.2) using 1X cell culture lysis reagent (Promega). Protein samples were quantified by Bradford's assay with the BIO-RAD protein assay (Biorad). Fifteen to sixty micrograms of each sample was boiled in sodium dodecyl sulfate (SDS, Sigma) sample buffer and run on 10-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein samples separated according to molecular weight were transferred to nitrocellulose membranes (GE Healthcare Amersham Hybond ECL). These membranes were blocked by incubation with 1X tris buffered saline with tween 20 (TBS-T) buffer containing 5% non-fat milk (block solution) for 1 h at 4°C. Primary antibodies, diluted in block solution, were added to the membranes and incubated overnight at 4°C. The next day, membranes were washed 5X with TBST and subsequently incubated for 1 h with either anti-mouse or anti-rabbit (GE Healthcare) horseradish peroxidase (HRP)-conjugated secondary antibodies that had been diluted in block solution. Protein bands were visualized by the ECL or ECL Plus Western

Blotting detection system (GE healthcare Amersham Hyperfilm ECL). Antibody information is provided in appendix Table 6.2.

2.2.11 Image acquisition and densitometry analysis

Films were scanned at a resolution of 600 dots-per-inch, set to grayscale, cropped and used to make figures. No software adjustments were made. Densitometry was performed on several exposures of scanned films to quantify relative protein levels using ImageJ (National Institute of Health) software.

2.2.12 Statistical analysis

All experiments were performed independently at least three times and representative data are presented in the figures. Means and standard deviation were calculated. Statistical difference between samples was evaluated with a Student's t-test. A P-value less than 0.05 was considered to be statistically significant. Other obvious increases/decreases were noted but clearly described as not significant.

2.3 Results

2.3.1 hPygopus2 mRNA and protein is expressed in a cell cycle dependent manner in MCF7 cells

The relative expression levels of hPYGO2 in different phases of the cell cycle were assessed by immunoblot in MCF7 BrCa cells. MCF7 cells were isolated from a 69 year old Caucasian patient and established as the first breast adenocarcinoma cell line at the Michigan Cancer Foundation in 1973 (Soule et al., 1973). It is the most commonly used cell line model for endocrine responsive BrCa. To obtain cells in each phase of the cell cycle I utilized the serum deprivation and release method (Pollack et al., 1990). MCF7 cells cultured in media supplemented with serum were collected and used as the unsynchronized cell samples (two samples collected). Serum deprivation was achieved by removing the serum supplemented media and growing cells in serum free media. Two G₀-phase cell samples were collected after this period of incubation. At the end of this interval, media containing serum was added and cells were grown for 57 h. Throughout this period, two cell samples were collected at each of the various time points. One cell sample (per time point) was processed and analyzed by flow cytometry, which identified the cell cycle phase based on DNA content. This enabled me to determine the cell cycle phase distribution within each sample for all the time points (Figure 2.1 A). From these data I determined that the approximate cell cycle length of MCF7 cells is 1 d. Furthermore, I identified time points that contained the highest proportion of cells in each cell cycle phase. The 39 h sample had the highest proportion of cells in G1 phase (60.57%), the 24 h sample had the highest proportion of cells in the S phase (69.47%)

and the 33 h time point sample was used to represent cells in the G2/M phase (31.85%) (Figure 2.1 B).

The second cell sample (for each time point) was processed for immunoblot analysis. More specifically, I only processed the samples that were identified as representative of each phase, based on the flow cytometry analysis.

hPYGO2 protein is detected as two differentially migrating bands, possibly identifying two post-translationally modified isoforms. Relative to β -Actin levels, hPYGO2 protein levels were highest in the G1 phase of the cell cycle, lowest in the G₀ phase and moderate in the S and G2/M phases of the cell cycle (Figure 2.1 C). The protein levels of hPYGO2 were slightly, but not significantly higher in the G2/M phase relative to the S phase.

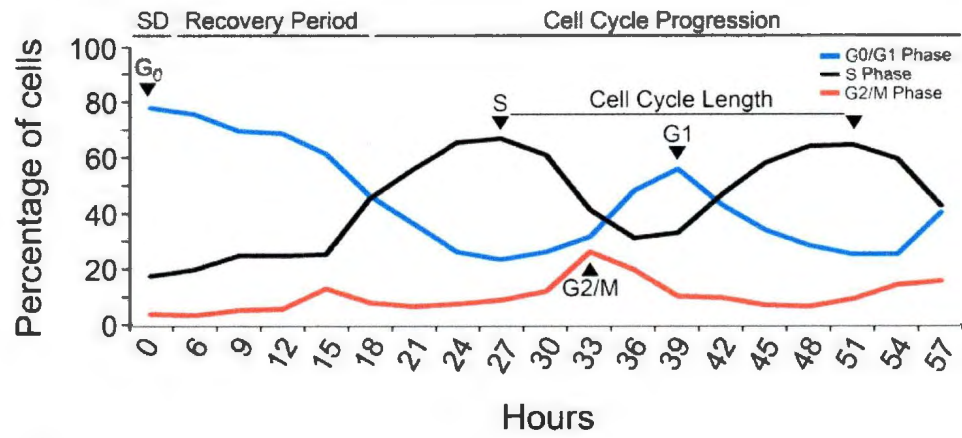
I also assessed the protein levels of some well-established cell cycle phase specific markers as a positive control. The expression of CCND was highest in the G1 phase which is consistent with previous results (Yang et al., 2006). PCNA has a role in DNA replication (Bowman et al., 2004) and is therefore highest in the S phase of the cell cycle (Leonardi et al., 1992), which was what I observed. Histone H3 (HH3) is exclusively phosphorylated (pHH3) in metaphase (Hans and Dimitrov, 2001), which was when its expression was highest in my analysis.

These results suggested that hPYGO2 protein exhibits a cell cycle dependent pattern of expression. However, one of the limitations of this approach was that the proportion of cells in certain phases was not very high. For example, in all my samples the highest percentage of cells in G2/M was 31.85%. This technical issue also explains

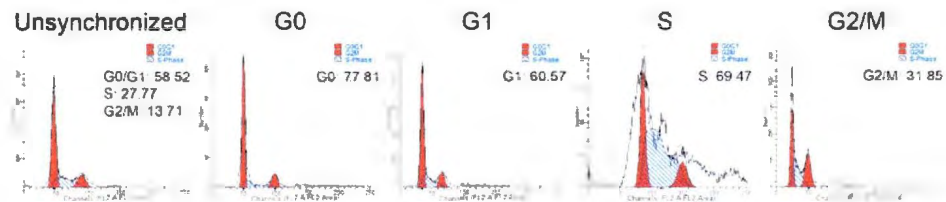
Figure 2.1 Cell cycle dependent expression of hPYGO2 protein in MCF7 cells.

(A) Percentage of MCF7 cells in each phase of the cell cycle after serum deprivation (SD) and at each time point after release. The time points at which the representative sample for each phase of the cell cycle (G₀, G₁, S and G₂/M) was collected are indicated with arrowheads. The method by which MCF7 cell cycle length was deduced is included. (B) Cell cycle distribution plots of representative samples of each phase of the cell cycle. (C) Expression levels of hPYGO2 and other cell cycle phase protein markers [Cyclin D, Proliferating cell nuclear antigen (PCNA) and phosphor-Histone H3] were measured by immunoblot in MCF7 cells. β -Actin was used as a loading control. Molecular weight is expressed in kilodaltons (kDa).

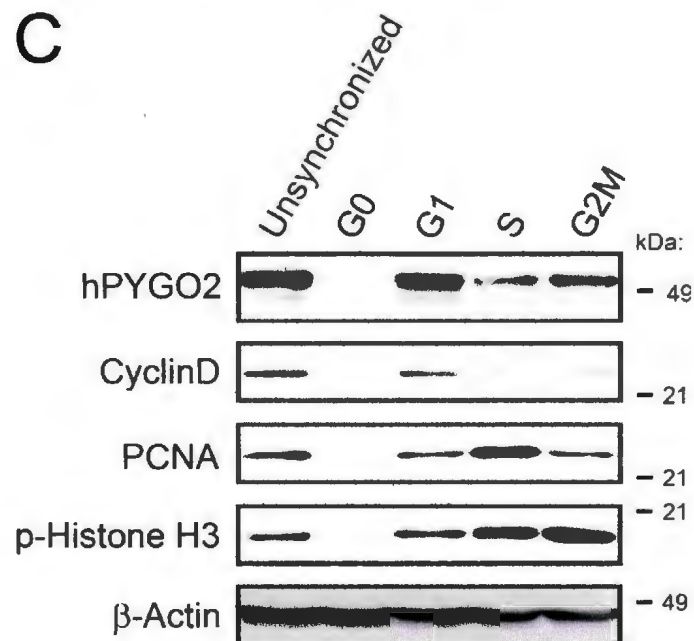
A



B



C



why hPYGO2 protein levels in the unsynchronized and G1 samples are almost equal. The percentage of cells in G1 in both samples is almost equal.

The relatively lower proportion of cells in the S and G2/M phases, compared to the G₀ and G1 phases, and the necessity to confirm my previous observations prompted me to obtain MCF7 cells in these phases by alternate methods. As I obtained high proportions of cells in the G₀ and G1 phases by the serum deprivation and release method, I elected to use this approach for acquiring cells in these phases.

To obtain cells in the S phase, I utilized the double thymidine block. As thymidine is a modified nitrogenous base, uptake by cells causes a disproportionate amount of one type of nucleotide relative to other types (Harper, 2005). The cell senses this imbalance during DNA replication and induces cell cycle arrest in the S phase. Treatment with paclitaxel was used to obtain cells in metaphase (Harper, 2005). Paclitaxel binds to tubulin subunits and hyperstabilizes microtubules, thereby preventing the physical separation of cells and arresting them at this phase (Harper, 2005).

MCF7 cells were subjected to the serum deprivation and release method and cells in the G₀ and G1 phases were isolated according to the time point parameters identified in the previous section. The double thymine block and paclitaxel (taxol) treatment was used to obtain cells in the S and G2/M phases, respectively. These protocols were also followed using thymidine and taxol vehicle controls (dH₂O and DMSO, respectively). Three samples were collected for each phase and used for cell cycle phase confirmation by flow cytometry (Figure 2.2 A), RNA extraction and mRNA expression analysis and

protein extraction and analysis. Distribution plots for the representative samples confirmed cell cycle arrest in the appropriate cell cycle phase (Figure 2.2 A)

The changes in *hPYGO2* mRNA expression throughout the cell cycle were assessed by Q-PCR. Neither of the vehicle controls affected *hPYGO2* mRNA levels, as they were not significantly different from the unsynchronized cell sample (Figure 2.2 B, top). *hPYGO2* mRNA levels were highest in the G1 phase of the cell cycle ($p=0.045$) and lowest in the G₀ phase of the cell cycle ($p<0.01$). The mRNA levels of *hPYGO2* in the S and G2/M phases were not significantly different from each other.

The changes in hPYGO2 protein levels closely paralleled the mRNA levels, being highest in G1, lowest in G₀ and approximately equal in S and G2/M phases (Figure 2.2 B, bottom). Additionally, the vehicle controls did not change hPYGO2 protein levels to the unsynchronized cell sample.

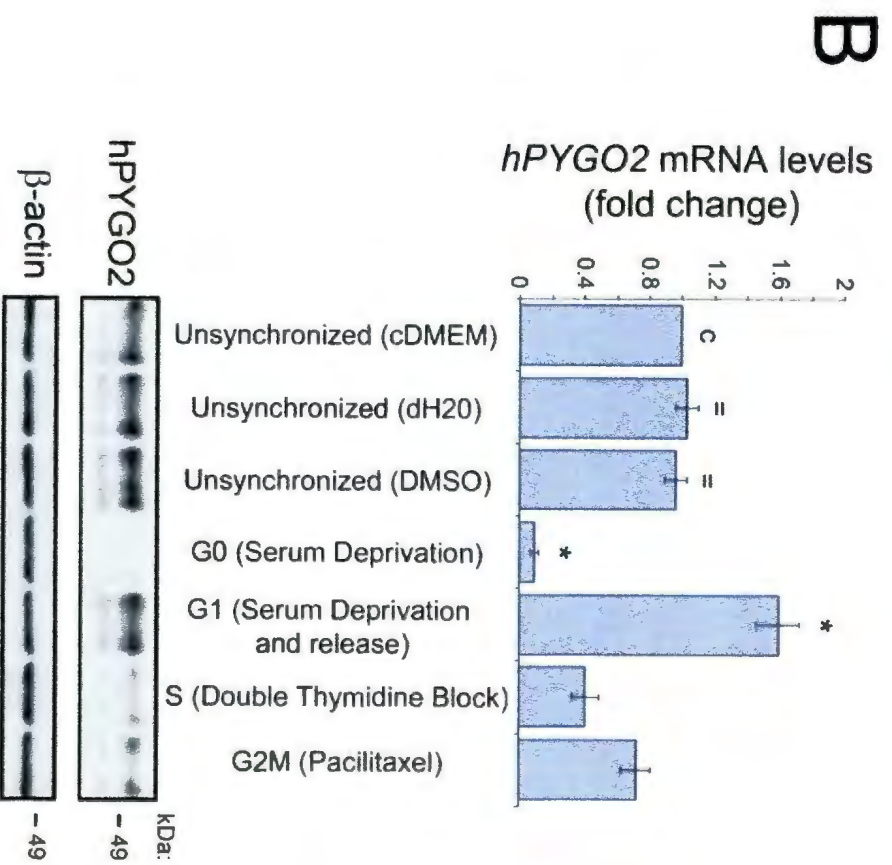
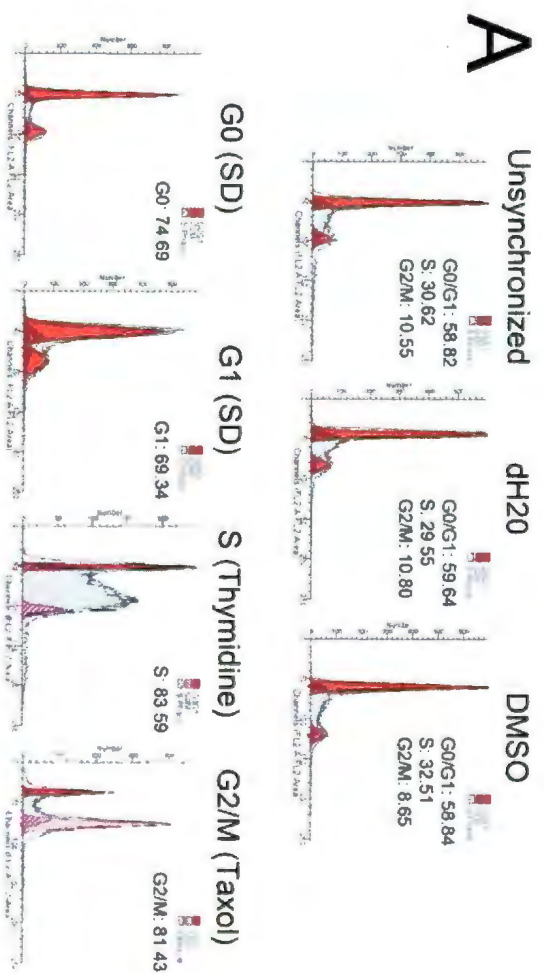
These results suggested that both hPygopus2 mRNA and protein are expressed in a cell cycle dependent manner in MCF7 cells and support the results obtained earlier (Figure 2.1 A). Furthermore, these findings suggest that *hPYGO2* may be primarily regulated at transcriptional level.

2.3.2 hPygopus2 protein exhibits cell cycle dependent expression in several cell lines

To determine if the cell cycle dependent expression of hPygopus2 was specific to MCF7 cells or if it was a general trend exhibited by several cell lines, I subjected five different cell lines to the serum deprivation and release method. The cancer cell lines included HeLa (cervical), MDA-MB-231 (ER α - breast), SKOV-3 (ovarian), PC-3

Figure 2.2 Cell cycle dependent expression of *hPYGO2* mRNA in MCF7 cells.

(A) Cell cycle distribution plots of representative samples of each phase of the cell cycle including the unsynchronized cells, vehicle control (dH₂O and DMSO) treated cells, serum deprivation and release method obtained G₀ and G₁ cells, double thymidine block S phase arrested cells and taxol treated G₂/M arrested cells. (B, top) Expression levels of *hPYGO2* mRNA as analyzed by Q-PCR in MCF7 cells during different phases of the cell cycle. Levels were normalized to β -Actin, set to 1 in the unsynchronized sample and adjusted accordingly in vehicle treated, serum deprived and released, thymidine and paclitaxel treated samples. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=). (B, bottom) Expression levels of *hPYGO2* protein as analyzed by immunoblot in MCF7 cells during different phases of the cells cycle. β -Actin was used as a loading control and molecular weight is expressed in kDa.



(androgen insensitive prostate) and LNCaP (androgen sensitive prostate) and the normal cell line was HEK293 (kidney). HeLa cells were cultivated in 1951 from an African American patient suffering from a particularly aggressive form of cervical adenocarcinoma (Scherer et al., 1953). MDA-MB-231 cells were isolated from a pleural effusion of a 51 year old patient with breast adenocarcinoma and are the most widely used ER α - BrCa cell line model (Cailleau et al., 1974). SKOV-3 cells were derived from metastatic ascites from a patient with ovarian adenocarcinoma (ATCC Number HTB-77). PC-3 cells were established in 1979 from the bone metastasis of a 61 year old patient with prostate adenocarcinoma (Kaighn et al., 1979). These cells are unresponsive to androgen treatment. The LNCaP cell line is androgen sensitive and was established in 1977 from a lymph node metastasis of a prostate adenocarcinoma patient (Horoszewicz et al., 1983). HEK293 cells were generated by transformation of normal human embryonic neuronal kidney cells with sheared adenovirus 5 DNA (Shaw et al., 2002).

These cell lines were processed identically to MCF7 cells except that samples for HeLa, HEK293 and MDA-MB-231 cell lines were collected for 51 h and samples from PC-3, SKOV-3 and LNCaP were collected for 3 d. This was due to the different cell division rates of these cell lines.

Confirmation of progression through the cell cycle was achieved by flow cytometry as described earlier (Section 2.3.1). Distribution plots were used to identify phase representative samples (appendix Figure 6.1) and the approximate cell cycle length for each cell line. Cell samples representative of each cell cycle phase were processed for immunoblot analysis.

Overall, the phase specific variation in expression of hPYGO2 in all of the cell lines examined was consistent with that observed in the MCF7 cells (Figure 2.3 A-F). hPYGO2 expression was highest and lowest in the G1 and G₀ phases, respectively. hPYGO2 protein levels in S and G2/M phases was not significantly different for all cell lines, except for LNCaP cells in which G2/M protein levels were significantly lower than in S.

Interestingly, in the relatively faster growing cell lines (HeLa, HEK293 and MDA-MB-231) there was a high level of hPYGO2 protein in the G2/M phase relative to the level in G1. In the relatively slower growing lines (MCF7, PC-3, SKOV-3 and LNCaP) the hPYGO2 protein level in G2/M was much lower than that in G1. In order to determine the exact levels of relative expression, I used densitometry to quantify hPYGO2 protein levels in the G1 phase and in the G2/M phase (Figure 2.4). For the fast growing cell lines, hPYGO2 levels were 5.1-11.1% lower in G2/M relative to G1. In contrast, for the slow growing cell lines, hPYGO2 levels were 26.2-46% lower in G2/M relative to G1 and significantly lower than the fast growing cell lines ($p=0.013$). These analyses supported the hypothesis that the percentage of hPYGO2 protein in G2/M relative to the percentage in G1 was inversely proportional to cell cycle length.

These results suggested that the cell cycle dependent expression pattern of hPYGO2 is a general trend. Additionally, the inverse correlation between cell cycle length and percentage of hPYGO2 in G2/M relative to G1 suggested that hPYGO2 may serve as a marker for cell proliferation rate.

Figure 2.3 Cell cycle dependent expression of hPYGO2 protein in several cell lines.

Expression levels of hPYGO2 protein as measured by immunoblot in HeLa (A), PC-3 (B), HEK293 (C), SKOV-3 (D), MDA-MB-231 (E) and LNCaP cells (F) in different phases of the cell cycle. β -Actin was used as a loading control and molecular weight was measured in kDa in all cases.

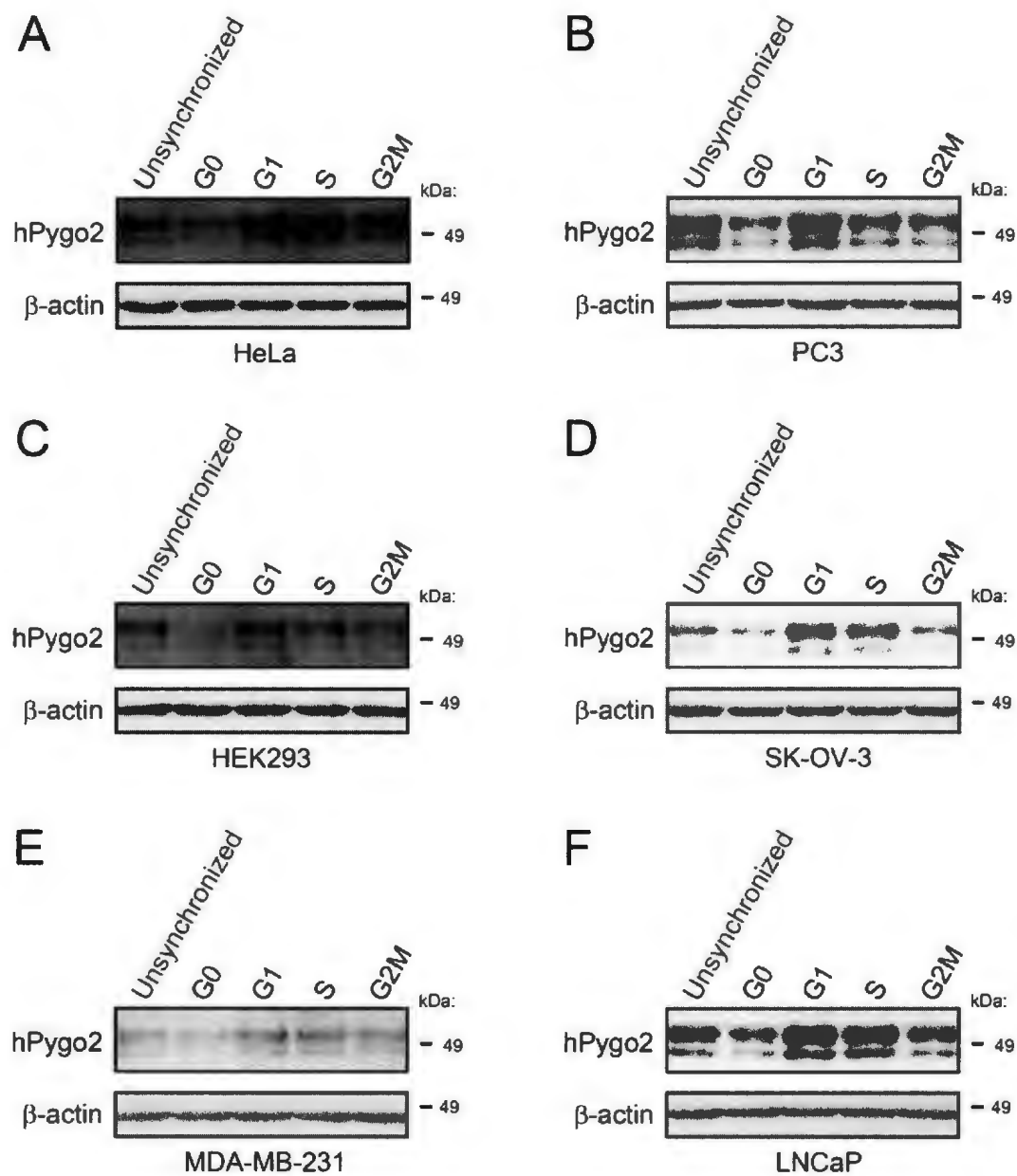
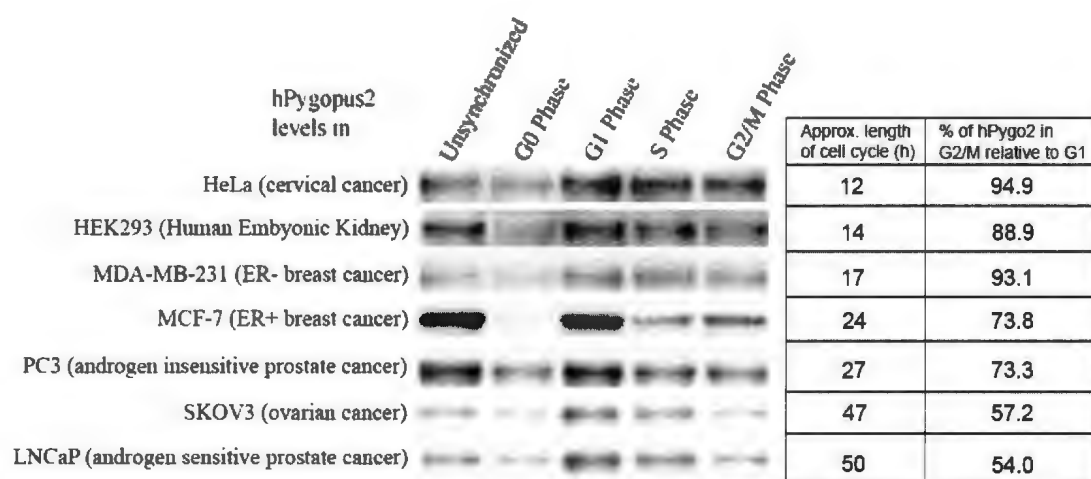


Figure 2.4 Inverse correlation between cell cycle length and cell cycle phase relative hPYGO2 protein levels.

Expression levels of hPYGO2 protein as measured using immunoblots in different cells lines aligned with the approximate cell cycle length of each cell line and the level of hPYGO2 protein in G2/M phase relative to G1 phase as determined by densitometry.



2.4 Discussion

The cell cycle dependent expression of hPygopus2 mRNA and protein suggested that *hPYGO2* is primarily regulated at the transcriptional level and that the proteins responsible for its expression exhibit cell cycle dependent activity. These observations are consistent with previous results demonstrating ELF1 regulation of *hPYGO2* (Andrews et al., 2008) and the hypothesized involvement of the RB protein. hPygopus2 levels were highest in the G1 phase which correlates with the inhibition of RB and transcriptional activation of ELF1. Furthermore, the observation that hPygopus2 cell cycle dependent levels are consistent between normal and cancer cell lines suggested that the RB-ELF1 pathway may be the predominant mechanism by which *hPYGO2* is regulated.

The observation that hPYGO2 is highest in G1 suggested that it may have a functional role during this phase. This is consistent with multiple previous results showing that knockdown of hPYGO2 protein causes an arrest in the G1 phase (Popadiuk et al., 2006; Andrews et al., 2007; Chen et al., 2010; Wang et al., 2010; Chen et al., 2011). Whether the required function of hPYGO2 is in Wnt signalling (Thompson et al., 2002), chromatin remodeling (Jessen et al., 2008), ribosomal DNA transcription (Andrews et al., Accepted) and/or another novel process, it is not unreasonable to think that a reduction of hPYGO2 protein levels would inhibit the(se) specific process(es) and thereby prevent the G1/S transition.

All of the above mentioned processes are involved in, or required for, the induction of gene expression during the G1 phase of the cell cycle. The absence of

hPYGO2, a transcriptional activator/chromatin remodeler, at such a time, would prevent critical genes from being expressed and thus disable transition through G1/S.

The cell cycle dependent expression of hPYGO2 also sheds light on the variability of hPYGO2 protein levels in tumour cells. While overexpression of hPYGO2 in tumours has clearly been established, its levels are not distributed uniformly within and between tumours. This could be due to unsynchronized cell populations within tumours (Weinberg, 2007d). Cells that exhibit the lowest levels or absence of hPYGO2 may be in the G₀ phase, while cells in which hPYGO2 expression is highest may be in G1. Cells expressing moderate levels of hPYGO2 may be in S or G2/M phases of the cell cycle.

The second major finding in this study was that the hPYGO2 protein levels in the G2/M phase relative to its levels in G1 are inversely correlated with cell cycle length. This finding, in combination with the relatively long half-life of the hPYGO2 protein (approximately 40 h, data not shown), led to the hypothesis that hPygopus2 is induced during the G1 phase and its protein is turned over progressively throughout the cell cycle. This could explain why faster growing cell lines (shorter cell cycle length) have more hPYGO2 in G2/M relative to that in G1. The relative hPYGO2 levels between G1 and G2/M phases could be utilized to determine tumour cell proliferation rate.

Tumour cell proliferation rate is a combination of the percentage of proliferating cells within a tumour and their cell cycle length (Steel, 1967). It is a very important prognostic and predictive factor (Tubiana, 1989; Kopper, 2001) and is used to determine disease progression and to select specific chemotherapy regimens (Tubiana, 1989; Ribba et al., 2009). In the clinic, determination of tumour cell proliferation rate is based on the

percentage of proliferating cells within a specific cell cycle phase (Tubiana, 1989). For example, the mitotic index, the current gold standard method to evaluate tumour cell growth rate, is a ratio of cells in mitosis relative to the total number of cells (Baak et al., 2009). The higher the number of cells in mitosis, the higher the tumour cell proliferation rate and the more aggressive the tumour is (Baak et al., 2009).

The limitation of mitotic index, and other such methods, is that they solely depend on the percentage of proliferating cells in one cell cycle phase and therefore do not account for tumour and host variability factors (for example proportion of proliferating cells, tissue damage, immune system, circadian rhythm) which will skew proliferation rate estimates (Kopper, 2001). Inaccurate determination of tumour aggressiveness and improper prescription of chemotherapeutic agents affects the patient psychologically (perception of diagnosis) and physically (survival) (Friberg and Mattson, 1997).

The inverse correlation between relative hPYGO2 protein levels and cell cycle length could allow hPYGO2 to be used as an accurate prognostic proliferation biomarker. The advantage of using hPYGO2 expression to determine tumour cell proliferation rate is that it is a ratio between two phases (doesn't rely solely on one cell phase) and therefore serves as an internal control for every tumour. The higher the level of hPYGO2 protein in G2/M relative to G1, the shorter the cell cycle length, the faster the proliferation rate and thus, the more aggressive the tumour.

Examination of hPygopus2 expression has an additional advantage because it may also identify the proportion of proliferating cells within a tumour. hPygopus2 expression is essentially non-existent in serum deprived cells, which should be equivalent to cells

within necrotic and hypoxic regions of the tumour. As hPygopus2 is overexpressed in cancer cells, its levels would be much higher in proliferating cells relative to non-proliferating cells.

The next step in this study would be to stain tumour sections to evaluate the accuracy of using hPYGO2 as a proliferation biomarker. This would involve indentifying the tumor cells in G1 and G2/M and quantifying relative levels of hPYGO2 between them. The obvious problem associated with this is the lack of a positive control as current methods cannot be used due to lack of accounting for the factors described above. Perhaps the best approach would be to generate tumors by xenografting cells in mice. This would be advantageous because the approximate time of tumor initiation would be known. Alternatively, a retrospective study could be performed by examining hPYGO2 expression on tumours that were established as slow and fast growing.

Additionally, the cell cycle dependent expression of *hPYGO2* mRNA may also be explored as a marker for tumour cell proliferation rate. The use of hPygopus2 mRNA versus protein would depend on the cell cycle length. Protein could be used for longer cell cycle lengths' due to its longer half-life and mRNA could be used for shorter cell cycle lengths' due to its shorter half-life.

**Chapter 3: 17 β -estradiol enhances hPygopus2 expression via Estrogen
receptor- α and SP1 transcription factor promoter complexes**

3.1 Introduction: Breast Cancer

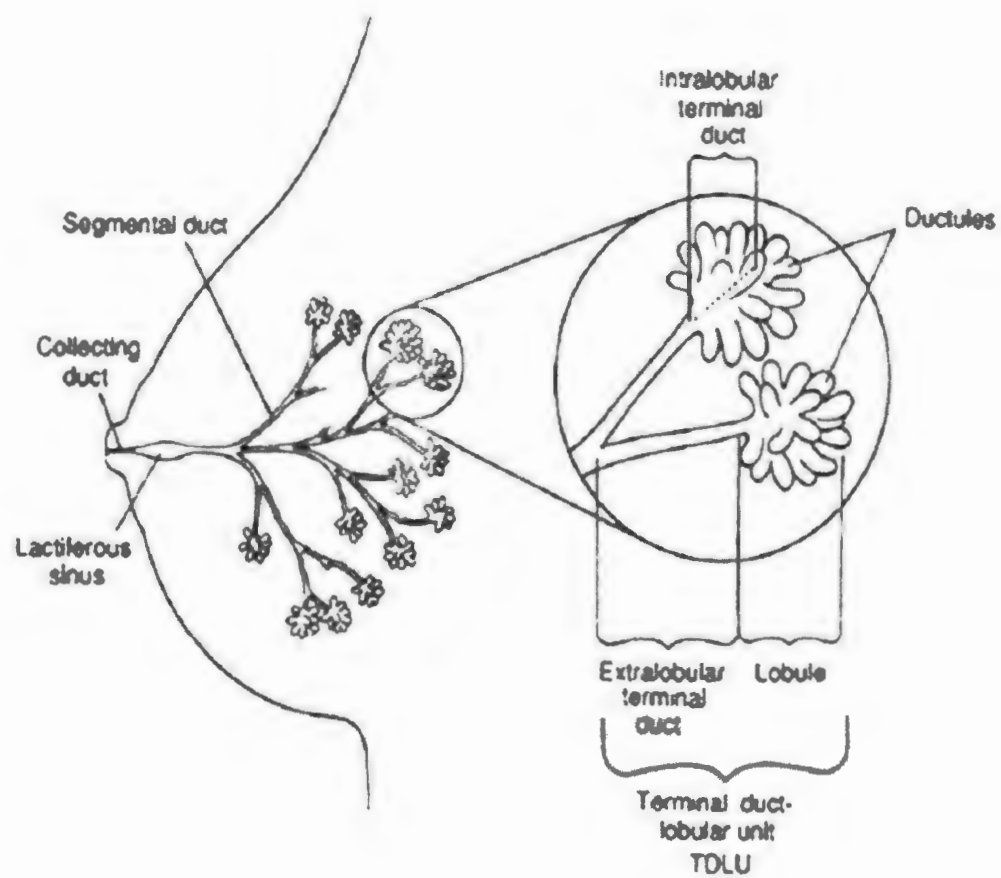
3.1.1 Foreword

In the previous chapter I characterized the cell cycle dependent expression of hPygopus2 mRNA and protein in several cancer cell lines. In particular, my evidence pointed to a role for hPYGO2 in the G1 phase. Identification of factors responsible for *hPYGO2* regulation would not only provide a better understanding of the signalling networks at play in cancer, but would also highlight additional ways by which hPygopus2 can be therapeutically targeted. Our lab has previously determined that ELF1 has a role in inducing *hPYGO2* (Andrews et al., 2008). However, the requirement of hPYGO2 during the G1/S transition, its cell cycle dependent expression in several cell lines and its hypothesized link to RB suggests that hPYGO2 may have a more fundamental link to the cell cycle. Thus, I decided to test if mitogenic stimulation would cause up regulation of *hPYGO2*. I selected to test my hypothesis in MCF7 BrCa cells as hPYGO2 has been previously examined in these cells and to use 17 β -estradiol as the mitogen as it has a predominant role in BrCa (and particularly in MCF7 cells).

3.1.2 The breast and breast cancer

The human adult mammary gland is comprised of a progressive branching glandular ductal system of 15–25 lobes in a fat pad (Hennighausen and Robinson, 2005) (Figure 3.1). Each lobe (also known as terminal duct lobular unit) originates at the nipple and is composed of a single terminal duct which branches to form ductules that are

Figure 3.1 Schematic diagram showing the hierarchical structure of the breast.



capped off by lobules, where milk is produced. The ducts, ductules and lobules are lined by a single cell thick epithelial layer of low columnar and cuboidal cells (Sgroi, 2010).

The mammary gland undergoes dramatic changes in size, shape and function throughout life (Ali et al., 2011). The main spurt of growth occurs during puberty and is dependent on high levels of E_2 . Post-pubertal development results in cyclical increases of extensive ductal branching in the fat pad. Estrogens maintain the ducts and promote the dynamic changes in side branching that occur during the menstrual cycle (Hennighausen and Robinson, 2005).

While there are several risk factors [genetics (ethnicity and family history), reproductive factors (breast density, age at menarche, parity and breastfeeding) and lifestyle (body mass index, physical activity, alcohol, smoking)], excessive estrogen exposure, and the resulting increased estrogen signalling, is one of the most predominant factors for the development of BrCa (Nelson et al., 2012). Furthermore, at least 70% of breast cancers are Estrogen receptor alpha ($ER\alpha$ or $ESR1$) positive (+) (Ali et al., 2011) and their growth is E_2 regulated. Both $ER\alpha$ overexpression (Khan et al., 1998) and genomic region amplification (6q25) (Holst et al., 2007) in the benign breast has been associated with an increased BrCa risk.

The progression from the normal breast to malignant cancer is a multistep process in which the exact cause and cell of origin (suspected to be a stem or progenitor cell) are currently unknown. The general depiction that emerges for the most common BrCa types, invasive ductal carcinoma (up to 80% of all breast cancers) and invasive lobular carcinoma (5-15%) (Weigelt and Reis-Filho, 2009), is that these cancers all originate in

the terminal duct lobular unit and progress from an initial hyper-proliferative stage to a precancerous, *in situ* carcinoma stage and then to invasive BrCa (Perou et al., 2000; Weigelt and Reis-Filho, 2009).

3.1.3 *17 β -estradiol signalling components*

Estrogens are produced by ovaries (and to a lesser extent the adrenal cortex) and secreted into the bloodstream, which is how they make their way to target organs. *17 β -estradiol* is the most potent of the three estrogens produced in the body (Kuiper et al., 1997).

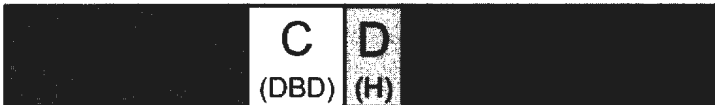
Even though E_2 was identified in the 1920's (Allen and Doisy, 1983), $ER\alpha$ was not identified until the 1960's (Toft and Gorski, 1966) and a second ER isoform, ER beta, was not discovered until 1996 (Kuiper et al., 1996). Because the role of ER beta in epithelial cancer cells is unclear, the following discussion will focus on and refers to $ER\alpha$.

Sequence similarity between the DBDs of $ER\alpha$ and the glucocorticoid receptor (Thornton, 2001) helped to classify this family of transcription factors within the nuclear receptor superfamily (Chawla et al., 2001). In addition to its highly conserved DBD, $ER\alpha$ has five defined functional regions (Herynk and Fuqua, 2004) (Figure 3.2).

The amino-terminal A/B regions contain an activation function 1 (AF-1) domain, which exhibits ligand-independent functions, and acts as a co-regulatory domain that is responsible for the recruitment of coactivators and corepressors. The C region features

Figure 3.2 Schematic diagram showing the conserved domains of ER α (generated from Akingbemi, 2005).

The A/B regions contain the activation function 1 domain (AF-1) which exhibits ligand-independent activation. The C region contains the DNA binding domain (DBD) which allows sequence specific DNA binding and homo- or heterodimerization. The D region has a flexible hinge and a nuclear localization sequence. The E/F regions contain the ligand binding domain (LBD), within which is the activation function 2 domain (AF-2). This portion exhibits different conformations depending on type of bound ligand and thereby promotes or hinders transcription activation.

ER α  Region
(Properties)

the DBD, which is comprised of two non-equivalent cysteine-rich zinc fingers (Green et al., 1988; Mader et al., 1989; Ruff et al., 2000). These zinc fingers are not only responsible for binding to estrogen response elements (EREs) and DNA tethering through other transcription factors, but also for receptor dimerization. The D region contains the hinge domain (H) and the nuclear localization sequence. The carboxy-terminal E and F regions contain the ligand-binding domain (LBD) - a wedge-shaped structure composed of 12 alpha-helices. Within the conformationally dynamic LBD is a ligand-dependent activation function 2 (AF-2) domain. These helices exhibit different orientations depending on the nature of the bound ligand and the cell-type specific profile of co-regulators (Katzenellenbogen et al., 1996; Katzenellenbogen and Katzenellenbogen, 2002; Norman et al., 2004; Shang, 2006). Additionally, they are responsible for the recognition of proteins that link ER α to the components of the general transcriptional machinery (Danielian et al., 1992; Wrenn and Katzenellenbogen, 1993; Henttu et al., 1997; Feng et al., 1998).

3.1.4 Classical genomic signalling by estrogens

In normal cells, 17 β -estradiol binding to the LBD of ER α causes intracytoplasmic chaperones (such as heat-shock proteins 70 and 90) to dissociate from the receptor molecule (Smith and Toft, 1993) and subsequently leads to the dimerization and nuclear translocation (Pace et al., 1997). Within the nucleus, ER dimers bind to sequence-specific palindromic EREs (5'-GGTCAnnnTGACC-3') (Klein-Hitpass et al., 1986). In consensus (perfect) ERE motifs each ER of the dimer binds to half of the ERE sequence.

Once bound to DNA, the E₂ stabilized receptors adopt an optimal conformation that is efficient for interaction with the steroid receptor coactivators (the three best characterized families include nuclear coactivator 1, 2 and 3) (Glass and Rosenfeld, 2000). The multi-protein coactivator complex is fully formed after recruitment of co-regulatory proteins, such as histone acetyltransferases, ubiquitin ligases and chromatin remodelers. Recruitment of coactivators serves to enhance receptor/basal transcription machinery interactions to efficiently activate transcription (Beato, 1991; Horwitz et al., 1996; Glass et al., 1997; McEwan et al., 1997; Shiama, 1997; Shibata et al., 1997).

After the initiation of transcription, post-translational modifications, such as methylation and acetylation, promote the dissociation of the complex, and the simultaneous ubiquitylation of ERs either results in further activation of the receptors or induces their degradation (Heldring et al., 2007). The ER and its coactivators have been shown to repeatedly cycle on and off several times in a predictable temporal manner (Shang et al., 2000; Metivier et al., 2003). While E₂ stimulates transcription of target genes via ER α , TMX and FUL have the opposite effects.

3.1.5 The mechanism of action for ER α antagonists

The demonstration that estrogens could promote mammary tumour formation led to the proposal that ER α antagonists could be used to treat BrCa. Thus TMX and several other related antiestrogens, particularly fulvestrant, were developed and are the first line adjuvant agents for the treatment of ER α + BrCa (Ali et al., 2011).

When four-hydroxytamoxifen (4-OHT), the active metabolite of TMX, binds to the LBD of ER it induces a repressive structural rearrangement that blocks access to the ER coactivators (Brzozowski et al., 1997; Shiau et al., 1998; Celik et al., 2007). As a further repressive mechanism, 4-OHT also alters the structure of ER such that it preferentially interacts with corepressor nuclear receptor boxes (Hu and Lazar, 1999) of corepressor proteins including Nuclear corepressor 1 and 2 (Huang et al., 2002; Horlein et al., 1995; Chen and Evans, 1995). These corepressors function as adaptors to recruit proteins that possess histone deacetylase activity (Keeton and Brown, 2005) and thereby inhibit transcription (Shang et al., 2000). The other ER α antagonist, FUL, has an even more potent inhibitory mechanism.

Following binding to ER, FUL blocks dimerization of the receptor and limits its nuclear translocation (Fawell et al., 1990; Dauvois et al., 1993; Robertson et al., 2001). Furthermore, FUL contains a bulky side chain that occupies the coactivator binding pocket and leads to an unstable receptor conformation making it more susceptible to degradation (Dauvois et al., 1992; Osborne et al., 2004). Additionally, FUL blocks the recruitment of other coactivators via both transactivation domains (AF-1 and AF-2) to ER, which are essential for maximum activation and transcription of ER genes (Osborne et al., 1995). It is for these reasons that FUL has no agonist activity and is considered a pure antiestrogen.

Not only can ER molecules interact with themselves to produce homodimers, but they can also interact with other transcription factors to generate heterodimers (non-classical genomic signalling), adding another level of complexity (Heldring et al., 2007).

Thus it is not surprising that most E₂ target genes do not contain an ERE palindrome in their promoter but have non-palindromic ERE half-sites through which E₂ regulation is mediated (Anolik et al., 1995). ERs commonly form heteromeric complexes with the SP1 transcription factor (SP1) protein, the Nuclear factor of kappa light polypeptide gene enhancer in B-cell (NFKB) protein, and the activator protein 1 (AP-1) complex (Heldring et al., 2007). One recent microarray and RNA interference study suggested that about 60% of E₂ responsive genes (induced or repressed) are ER α and SP1 protein dependent (Wu et al., 2009).

3.1.6 SP1 transcription factor proteins

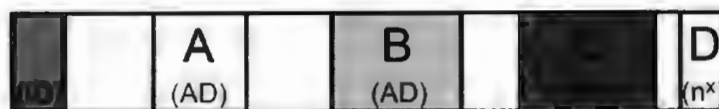
SP1 belongs to the Specificity Protein/Krüppel-like Factor transcription factor family (Suske et al., 2005). This family is characterized by the highly conserved DBD (domain C) which contains three adjacent Cys₂His₂-type zinc fingers. By utilizing their DBD, these proteins bind GC-boxes (consensus sequence GGGGCGGGG) (Letovsky and Dynan, 1989) in target gene promoters (Suske et al., 2005). In addition, SP1 has an N-terminal inhibitory domain, two transactivation subdomains (A and B) and a carboxy terminal domain (D) which is required for synergistic activation (Suske, 1999) (Figure 3.3).

SP1 proteins are ubiquitously expressed in mammalian cells and are especially important for differentiation, cell cycle progression and oncogenesis (Davie et al., 2008). The 12,000 GC-boxes in the human genome and their association with genes in virtually

Figure 3.3 Schematic diagram showing the conserved domains of SP1 (generated from Suske et al., 2005)

The N-terminal inhibitory domain (ID) can interact with corepressors to inhibit SP1 transcriptional activity. The A and B domains contain two transactivation subdomains (AD). The C domain contains the zinc fingers that enable sequence specific DNA binding. The D domain allows homomultimerization which enables synergistic activation.

SP1



Domain
(Properties)

all cellular processes (Cawley et al., 2004) provides a glimpse of the universality of SP1 proteins.

In cancer cells, the protein expression levels of SP1 are often greater than in normal cells. Specifically, in comparison to normal cells or tissues, SP1 levels are higher in breast (Zannetti et al., 2000) and hepatocellular carcinomas and in thyroid (Chiefari et al., 2002), pancreatic, colorectal, gastric (Kitadai et al., 1992; Jiang et al., 2004) and lung cancers (Davie et al., 2008; Chuang et al., 2009; Kong et al., 2010; Kong et al., 2011).

Abnormally high SP1 protein levels are correlated with poor prognosis and the stage of cancers (Wang et al., 2003; Yao et al., 2004; Safe and Abdelrahim, 2005). Moreover, inhibition or knockdown of SP1 to normal cellular levels decreases tumour formation, tumour growth and metastasis (Jiang et al., 2004; Lou et al., 2005; Yuan et al., 2007).

Functionally, in cancer, SP1 target genes include factors involved in cell cycle progression and arrest (Sherr and Roberts, 1999; Feng et al., 2000; Abdelrahim et al., 2002; Lagger et al., 2003), both pro- and anti-angiogenic factors (Yuan et al., 2007), pro- and anti-apoptotic factors (Kavurma et al., 2001; Kavurma et al., 2002; Kavurma and Khachigian, 2003), proto-oncogenes and tumour suppressors (DesJardins and Hay, 1993; Olofsson et al., 2007).

One way of targeting SP1 is by using chemotherapy agents that block binding to GC-boxes, and therefore, prevent SP1 target gene activation. Mithramycin A, curcumin, tolfenamic acid and betulinic acid are all chemotherapeutic agents that have such activity (Leask, 2012). Treatment with betulinic acid and curcumin reduced the expression and

activity of SP1 and resulted in lowered expression of SP1 target genes including Epidermal growth factor receptor, Cyclin D1 and Vascular endothelial growth factor (Wang et al., 1997; Kang and Chen, 2009; Chadalapaka et al., 2010).

3.1.7 ER α and SP1 complex formation, promoter binding and gene activation

As mentioned, ER α can form complexes with SP1 to modulate transcription of E₂ responsive genes. While there has been extensive research in this area, there is much discrepancy due to the methodology. An overall conclusion is that the binding of ER α to SP1 and the transcriptional effect of this interaction is cell type- (ER α ⁺ or ER α - BrCa or other cancerous or normal cells), protein level/type- (endogenous, transiently or stably overexpressed and full, partial or engineered sequence), ligand- (E₂, 4-OHT and FUL have similar activity on naked luciferase vector promoters) and promoter-dependent (EREs, ERE half-sites, GC-boxes and/or endogenous promoter segments on labeled oligos, reporter vectors and/or endogenous genes).

The loading of ER α onto E₂ responsive promoters may be direct or indirect, adding another level of complexity. Direct binding involves contact of both factors with DNA (Carroll et al., 2006; Menendez et al., 2010), while indirect binding involves the tethering of ER α to DNA through SP1 (O'Lone et al., 2004). In promoters with a consensus EREs or ERE half-sites, ER binding is likely direct. However, for other E₂ responsive promoters (which don't have an EREs or ERE half-sites) ER binding is most

likely indirect and through another transcription factor (e.g. SP1, NFkB or AP-1) (Carroll et al., 2005; Lin et al., 2007).

Somewhat unexpectedly, there appears to be no consistent pattern regarding the relative orientations or positions of ERE half-sites and GC-boxes. In most cases, the sequence GGTCA of the half-ERE is on the same strand as the GGCGGG sequence of the GC-box. However, the distance between the sites, as well as the position and strandedness of these elements relative to the transcription start site, can be completely variable (O'Lone et al., 2004). The general trends that have been observed concerning localization, interaction and promoter binding and activation are described below.

While ER α translocation to the nucleus depends on E₂ treatment, SP1 is a constitutively nuclear protein and its translocation is not affected by E₂ treatment (Dong et al., 2006). Due to the relatively low endogenous levels of these proteins, nuclear co-localization is only observed if the proteins are overexpressed. This co-localization is increased with E₂ treatment (He et al., 2005; Kim et al., 2005).

The endogenous interaction between ER α and SP1 requires E₂ and is blocked when cells are pretreated with FUL (Dong et al., 2006). In contrast, overexpression of ER α and SP1 allows these proteins to interact in the absence of E₂, but in this case the interaction is increased by E₂ treatment (Kim et al., 2005; Kang et al., 2011). The ER α interaction with SP1 doesn't require DNA binding (Kang et al., 2011), but is stabilised by the presence of DNA. The physical interaction between ER α and SP1 proteins is mediated by the AF-1 (Saville et al., 2000) or DBD (Kang et al., 2011) of ER α and the DBD of SP1 (Kang et al., 2011).

Binding to and activation of promoters by the ER α -SP1 complex depends on a combination of factors. Because of the different mechanism of action (direct or indirect/tethering), there is a requirement for either both the ERE half-site and GC-box or just the GC-box. When ER α and SP1 are overexpressed, due to their high levels and interaction, there is residual binding to promoters. However, this complex is inactive (Saville et al., 2000) and requires E₂ treatment for transactivation (He et al., 2005). While E₂ treatment does not increase SP1 promoter binding (Dong et al., 2006; Kang et al., 2011), it does cause nuclear translocation of ER α , which enhances binding of the ER α -SP1 complex to the promoter (He et al., 2005) and contributes to coactivator recruitment (O'Lone et al., 2004). The highest level of promoter binding is observed when both ER α and SP1 are overexpressed and treated with E₂ (Kim et al., 2005; Dong et al., 2006).

3.1.8 ER α target genes

From the discussion above, it is not surprising that E₂-ER regulated promoters/enhancers fall in two major categories: those in which ER directly binds DNA and those in which ER associates with regulatory sequences due to interactions with other DNA-binding proteins.

The first category can be further broken down into three subcategories based on the contents of the promoter: palindromic ERE sequences, ERE half-sites and ERE half-sites in proximity to GC-boxes. Human genes that contain perfect ERE sequence include Estrogen receptor binding antigen 9 (Watanabe et al., 1998), Trefoil factor 1 (Berry et al.,

1989), Tripartite motif containing 25 (Ikeda et al., 2000), Cathepsin D (*CTSD*) (from -145 to -101) (Cavailles et al., 1993; Krishnan et al., 1994; Wang et al., 1997) and Telomerase reverse transcriptase (Kyo et al., 1999). ERE half-sites are found in the promoters of ER α (Treilleux et al., 1997) and Prothymosin alpha (Martini and Katzenellenbogen, 2001). Direct DNA binding of both ER α -SP1 complexes has been shown to occur at the Transforming growth factor alpha (*TGFA*) (El-Ashry et al., 1996; Vyhldal et al., 2000), Retinoic acid receptor alpha (*RARA*) (Rishi et al., 1995) and *CTSD* (from -199 to -165) (Cavailles et al., 1993; Krishnan et al., 1994; Wang et al., 1997) promoters.

The second category, in which promoter binding of SP1 alone confers E₂ responsiveness, occurs in *CCND* (Sabbah et al., 1999; Castro-Rivera et al., 2001), Epidermal growth factor receptor (Salvatori et al., 2000), FBJ murine osteosarcoma viral oncogene homologue (*FOS*) (Duan et al., 1998), Heat shock binding protein 1 (Porter et al., 1997) and Adenosine deaminase (Xie et al., 1999).

One of the best characterized E₂ target genes in BrCa is *CTSD*, thus it has been commonly used as a positive control in the identification of novel E₂ target genes (Xing and Archer, 1998). The *CTSD* gene encodes a ubiquitous lysosomal aspartyl protease (Westley and May, 1996). Transcription from the *CTSD* gene is initiated at five sites but E₂ stimulates transcription via ER α and SP1 in the -199 to -165 promoter region (Cavailles et al., 1993).

It can be hypothesized that while E₂ induces the target genes discussed above, treatment with E₂ antagonists (4-OHT and FUL) will repress these genes.

3.1.9 ER α antagonists induce breast cancer cell death

TMX has been repeatedly shown to inhibit the growth of ER α -positive and E₂-sensitive BrCa cell lines (Lippman et al., 1976). This is associated with its ability to inhibit the expression of ER α target genes that regulate cell cycle and apoptosis (Musgrove and Sutherland, 2009). Specifically, repression of Cyclin D1, v-Myc myelocytomatosis viral oncogene homologue and B-cell CLL/lymphoma 2 genes and reduction of SP1 and NF κ B activity are among the events that account for the increased cell death that is observed in TMX-treated ER α -positive breast cancers (Butt et al., 2005; Musgrove et al., 2008; Nehra et al., 2010).

3.1.10 Nongenomic signalling by E₂ and ligand independent activation of ER α

In addition to the well-studied transcriptional effects of E₂ (classical and non-classical genomic signalling), steroid treated cells can respond by activating nongenomic signalling pathways (Checkis et al., 2007; Ellmann et al., 2009). These pathways are mediated by plasma membrane bound ER α (within other receptor complexes or caveolae) or endoplasmic reticulum membrane bound G-protein coupled receptor 30. Ligand binding to these receptors results in the rapid induction of cytoplasmic signalling cascades, such as Adenyl cyclase, Mitogen activated protein kinase and Phosphatidylinositol 3 kinase. These cascades frequently end with the activation of transcription factors that activate target gene transcription. As E₂ can activate gene expression without ER α , ER α can also activate gene expression without E₂. This consists

of growth factor activated cytoplasmic kinases, such as Protein kinase B, Phosphatidylinositol 3 kinase and Mitogen activated protein kinase, phosphorylating ER α , thereby translocating it to the nucleus where it can activate transcription.

3.1.11 Chapter summary

In chapter two I demonstrated that hPygopus2 expression is highest in the G1 phase of the cell cycle in several cancer cell lines, including the MCF7 ER α + cell line. I hypothesized that if *hPYGO2* regulation is directly linked to RB, then any mitogenic stimulus, which inactivates RB due to CCND expression, would cause the up regulation of *hPYGO2*. To test this hypothesis in BrCa cells, I studied the effect of 17 β -estradiol as the mitogen due to its predominant role in BrCa.

Sustained E₂ exposure plays a critical role in the initiation and progression of BrCa. The best characterized mechanism by which E₂ induces BrCa cell proliferation is through the ER α . The critical role of ER α in BrCa is clearly solidified by the fact that first line BrCa treatments include TMX and FUL, which function by binding to, and inhibiting the transcriptional activation abilities of ER α .

While the ER α commonly exerts its functions as a homodimer, it also interacts with other transcription factors, such as SP1. SP1 is a ubiquitously expressed protein, which is overexpressed in numerous cancer cell lines and tumour types. It has also been shown to be required for several aspects of oncogenesis, just like ER α . There are two similar, and thus fairly difficult to distinguish, mechanisms by which ER α -SP1

complexes can induce target gene expression. The direct method involves the binding of both ER α and SP1 to their respective promoter elements, the ERE half-site and the GC-box, respectively. The indirect method involves the tethering of ER α to DNA bound SP1.

In this study, I provide evidence for the E₂ enhanced expression of hPygopus2 through the direct binding of the ER α -SP1 complex to the *hPYGO2* promoter. My data indicated that even in ER α - BrCa, SP1 still plays a role in the regulation of *hPYGO2* and other “ER α -SP1” target genes. The finding that SP1 is required for the proliferation of ER α - BrCa cells suggests that hPYGO2 expression might assist in chemotherapy selection in endocrine disruptor unresponsive BrCa cells.

3.2 Materials and Methods

3.2.1 Cell maintenance

BT-20, BT-474, MDA-MB-157, MDA-MB-231, MDA-MB-468, SK-BR-3, MCF7, ZR-75 and T-47D BrCa cells (ATCC) were maintained in DMEM at 37°C with 5% CO₂.

VC-5 (derived from MDA-MB-231, ER α -) and MC-2 (derived from MDA-MB-231, wildtype ER α +) cell lines were generous gifts from Dr. Sheila Drover via Dr. V. C. Jordan. These cells are cultured in PRF-DMEM containing 5% dtFBS, 0.5 mg/ml G418, 6 ng/ml insulin and 20 mM L-glutamine. They were previously generated and characterized (Levenson and Jordan, 1994). The results of a preliminary analysis that I performed, which is consistent with previous results (Levenson and Jordan, 1994), is found in appendix Figure 6.2.

3.2.2 Drug treatments

Thirty thousand MCF7 cells per well were seeded in six well plates in cDMEM for 1 d. After this period, hormone deprivation was achieved by washing once with PBS and culturing in PRF-DMEM containing 2% dtFBS for 3 d. At the end of this period, the cells were washed once with PBS and incubated in PRF-DMEM containing 2% dtFBS. MC2 and VC5 cells were seeded at a density of 3×10^5 cells/well in six well plates for 1 d, according to the conditions described in Section 3.2.1. Drug treatments were carried out according to the conditions below.

For all analyses except luciferase assays, cells were treated with 10 nanomolar (nM) 17 β -estradiol (Sigma) (determined to be the most effective concentration) for the following times: 0.25, 0.5, 1, 4, 8 and/or 24 h. For the antiestrogens, 10 micromolar (μ M) of 4-OHT (Sigma) or FUL (Sigma) was used. The same volumes of ethanol (Et) and DMSO served as vehicle controls. When 4-OHT and FUL were used in combination with E₂, cells were pretreated with 4-OHT or FUL, or their vehicle controls, for 6 h. The concentration of cycloheximide (CHX) used was 50 μ g/ml (Schneider-Poetsch et al., 2010). For luciferase assays, 100 nM E₂ was used because this is the amount required to induce detectable levels of luciferase activity (Miralles et al., 1994).

3.2.3 RNA extraction, cDNA generation and Q-PCR

Performed as described earlier (Sections 2.2.8 and 2.2.9).

3.2.4 Protein extraction and immunoblotting

Performed as described earlier (Section 2.2.10).

3.2.5 hPygopus2 promoter analysis

The 1494 base pair (bp) sequence upstream of the *hPYGO2* gene transcriptional start site was pasted into the box on the Transcription Element Search System website (<http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME>). The putative binding sites identified by this software were examined with a specific focus on ERE palindromes, ERE half-sites, GC-boxes, AP-1 sites and NF κ B sites.

3.2.6 Plasmids

Luciferase reporter plasmids including pGL3-1494, pGL3-1143, pGL3-829, pGL3-531, pGL3-225 and the empty vector control pGL3-basic were previously described (Andrews et al., 2008). The ER α (pcER α) and SP1 (pcSP1) expression vectors and their empty vector control (pcDNA3.1) were all generous gifts from Dr. Paterno and described elsewhere (Ding et al., 2004; McCarthy et al., 2008). Vectors generated by site directed mutagenesis are described below.

3.2.7 Site directed mutagenesis

Luciferase vectors containing mutations in the GC-box at -356 (pGL3-531 mutGC), the ERE half-site at -331 (pGL3-531 mutERE), or both (pGL3-531 mutERE+GC) were generated by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis kit, Stratagene) according to the sequences in Letovsky et al., 1989 and Krishnan et al., 1994. The ER α (pcER α -DBM) and SP1 (pcSP1-DBM) DBD mutants were generated by site directed mutagenesis according to the sequences in DeNardo et al. (DeNardo et al., 2007) and information provided in Saegusa et al. (Saegusa et al., 1997), respectively. In both cases, amino acids which are required for the structural integrity of the first zinc finger were targeted. Primer sequences are listed in appendix Table 6.1.

3.2.8 Transient transfections

MCF7 cells were seeded and hormone deprived as described earlier (Section 3.2.2). They were subsequently transfected with combinations of expression vectors (empty vector, ER α , SP1 or ER α +SP1) and cotransfected with either one of the wildtype

hPYGO2 reporter constructs or with one of the mutated GC-boxes and/or ERE half-site reporter constructs and with a plasmid expressing β -galactosidase (pRSV- β -gal, β -gal). Cells cultured in six well plates were transfected with 1 μ g of total DNA. Cells cultured in ten centimeter dishes were transfected with 5 μ g of total DNA. Cells were treated with E_2 (as described in Section 3.2.2) and cultured for 1 d.

3.2.9 Luciferase and β -galactosidase assays

To measure luciferase activity, cellular protein was extracted using 1X cell culture lysis reagent (Section 2.2.10), combined with room temperature luciferase assay reagent (Promega, reconstituted as per the manufacturer's instructions) and read with the analytical Luminescence Laboratory Monolight 2010 machine.

β -galactosidase (β -gal) assays were performed by adding extracted cellular protein (described above) to β -gal buffer [100% Z-buffer (16.1 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5.5 g/l $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 0.75 g/l KCl and 0.246 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7), 0.27% β -mercaptoethanol (Sigma) and 4 g/l ortho-Nitrophenyl- β -galactoside (Sigma)], incubating at 37°C, stopping the reaction with 1 molar Tris (pH 11) and reading the absorbance (415 nanometers) on a Bio-Rad Model 3550 Microplate Reader. Luciferase activity of an empty well was subtracted and adjusted luciferase activity was normalized relative β -gal activity.

3.2.10 Chromatin immunoprecipitation

MCF7 cells were seeded and hormone deprived as described earlier (Section 3.2.2). Cells were transfected with wildtype or mutant *hPYGO2* promoter construct in

cases where binding to these reporter constructs was assessed. Otherwise cells were E₂, 4-OHT and/or FUL treated as described in Section 3.2.2 or treated with RNA interference (RNAi) as described in the following sections (Section 3.2.11 and 3.2.12).

Protein-DNA complexes were cross-linked with 0.75% formaldehyde for 10 m. Cross-linking was quenched by adding 125 mM glycine and cells were washed with PBS, harvested and re-suspended in lysis buffer [20 mM Tris-HCl (pH 8), 85 mM KCl, 0.5% Nonidet P-40] containing protease inhibitors and sonicated 8X for 10 second (s) pulses (20%) with 20 s breaks. DNA shearing to the correct size was confirmed by running samples on agarose gels. The soluble chromatin was collected by centrifugation and the supernatants were incubated with 50 µl of protein A or protein G slurry (50% slurry pre-cleared with 1 mg/ml bovine serum albumin and 0.4 mg/ml herring sperm DNA and re-suspended in TE buffer with 0.02% azide) under gentle agitation for 1 h at 4°C. The supernatant was transferred to a new tube, and 1-2 µg of antibody was added and samples were incubated overnight at 4°C (10 µl aliquots were removed before antibody addition to be used as input controls). Thirty microliters of 50% pre-blocked protein A or G bead slurry was added for 1 h. The pellets were successively washed 2X for 1 m in 1 ml low salt buffer [20 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100 and 0.1% SDS], 1 ml high salt buffer [20 mM Tris-HCl (pH 8), 500 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1% SDS], 1 ml LiCl buffer [20 mM Tris-HCl (pH 8), 250 mM LiCl, 1 mM EDTA, 1% Nonidet P-40 and 1% Na-deoxycholate] and 1 ml TE buffer [10 mM Tris-HCl (pH 8) and 1 mM EDTA]. Protein:DNA complexes were eluted in 190 µl elution TE buffer containing 1% SDS for

30 m, and the cross-links were reversed by overnight incubation at 65°C. Samples were equilibrated to room temperature and incubated first with 1 µl of 10 mg/ml RNaseA for 15 m at 37°C and then with 8 µl Tris-HCl (pH 6.5), 4 µl EDTA (500 mM) and 1 µl of 10 mg/ml Proteinase K for 1 h at 45°C. DNA was extracted using the DNeasy Tissue Kit (250) (Qiagen) and eluted in 50 µl of dH₂O. Promoter occupancy was assessed by Q-PCR as described in Section 2.2.9. Antibodies used for immunoprecipitation and primer sequences for amplification are listed appendix Table 6.2 and appendix Table 6.1, respectively.

3.2.11 RNA interference

The ELF-1 directed siRNA (siELF1) and the non-target control siRNA (siNTC) used were previously described (Andrews et al., 2008). An SP1 specific siRNA (siSP1) oligonucleotide was synthesized by Dharmacon according to the sequence in Abdelrahim et al. (Abdelrahim et al., 2002). Oligonucleotide sequences are listed in appendix Table 6.1.

3.2.12 siRNA transfections and rescue assays

Cells were seeded in six well plates and forward transfected with siRNA oligonucleotides at final concentrations of 10-25 nM using Lipofectamine RNAiMAX (Invitrogen) as per the manufacturer's instructions.

In rescue assays, cells were additionally transfected with 1 µg per well of the pCS2+ or pCS2+ELF1 expression vectors 1 d after the siRNA transfection as described

earlier (Section 3.2.11) using Lipofectamine with Plus Reagent (Invitrogen) as per the manufacturer's instructions. Cells were harvested 3 d after seeding.

3.2.13 Image acquisition and densitometry analysis

Performed as described earlier (Section 2.2.11).

3.2.14 Statistical analysis

Performed as described earlier (Section 2.2.12).

3.3 Results

3.3.1 *hPygopus2* protein expression in breast cancer cell lines

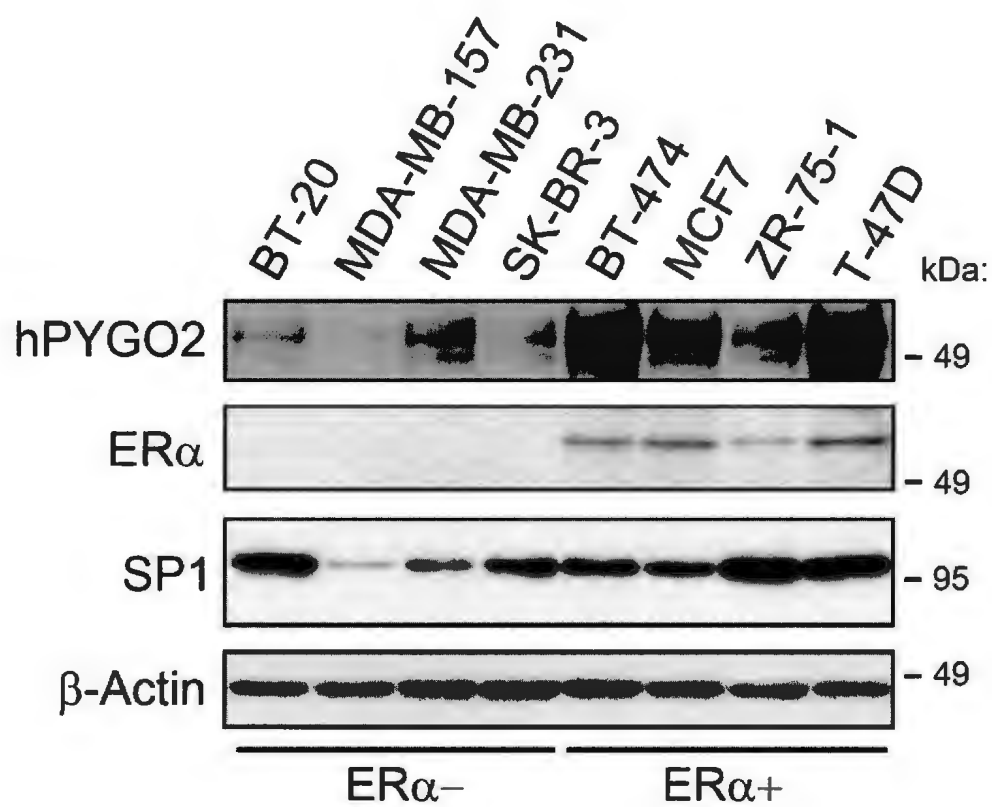
The relative expression of hPYGO2 protein in a variety of ER α - and ER α + BrCa cell lines was assessed by immunoblot. The status of the ER α - (BT-20, MDA-MB-157, MDA-MB-231 and SK-BR-3) and ER α + (BT-474, MCF7, ZR-75-1 and T-47D) cell lines was confirmed by blotting for ER α . hPYGO2 protein is detected as two differentially migrating bands, possibly identifying two post-translationally modified isoforms. Its expression was consistently higher in the ER α + cells relative to the ER α - cells (Figure 3.4). The relative expression of SP1, an important ER α interacting factor, was also measured. SP1 protein levels were lowest in MDA-MB-157 cells, slightly higher in MDA-MB-231 cells and highest and fairly uniform in the remainder of the cell lines. These observations suggested that hPYGO2 exhibits higher expression in ER α + relative to ER α - BrCa cells.

3.3.2 *17 β -estradiol* induces *hPygopus2* in Estrogen receptor alpha positive breast cancer cell lines

The higher levels of hPYGO2 observed in ER α + cells suggested that its expression may be modulated/enhanced by the ER α ligand, 17 β -estradiol (E₂). The effects of E₂ on hPygopus2 expression were evaluated in three BrCa cell lines. The MCF7 line was utilized as a model for hormone dependent BrCa, given that several E₂

Figure 3.4 Expression analysis of hPYGO2 in ER α ⁺ and ER α ⁻ BrCa cell lines.

Expression levels of hPYGO2, ER α and SP1 protein as measured by immunoblot in a panel of BrCa cell lines. β -Actin was used as a loading control and molecular weight is expressed in kDa.

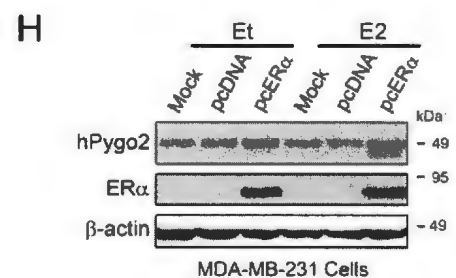
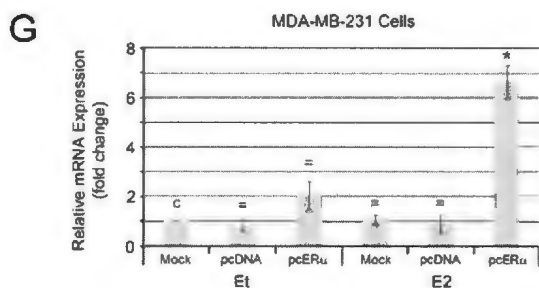
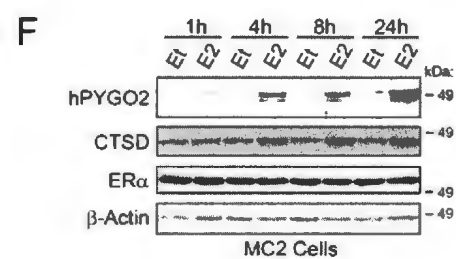
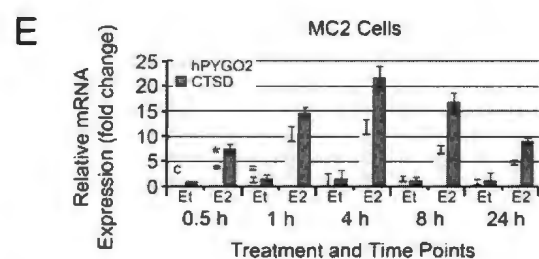
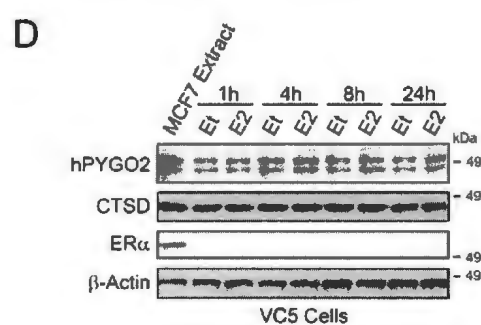
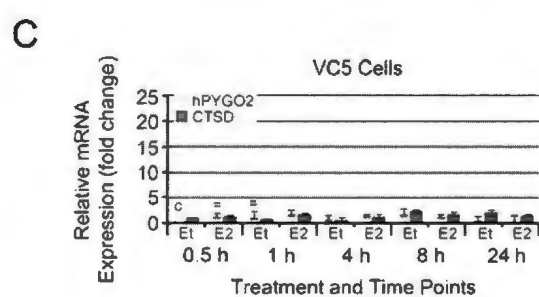
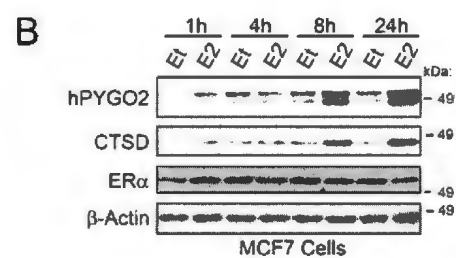
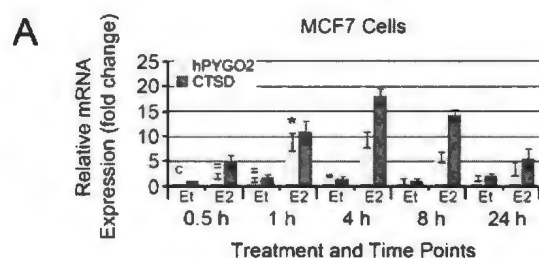


responsive target genes were characterized in these cells. The MC2 cell line is derived from MDA-MD-231 cells (ER α -) that were stably transfected with wildtype *ER α* cDNA under control of the CMV promoter and therefore constitutively expressed (Levenson and Jordan, 1994). I elected to use this cell line so that ER α expression within and between treated cells would remain uniform. VC5, the negative control cell line, was generated by stably transfecting MDA-MB-231 cells with an empty vector and therefore remains ER α - . Cell lines generated by the same methodology were extensively characterized previously (Levenson and Jordan, 1994). I measured the expression of a selection of genes (appendix Figure 6.1) to confirm the authenticity of the MC2 and VC5 cell lines.

MCF7, MC2 and VC5 cells were grown in the absence of E₂ for 3 d followed by either treatment with Et or 1 nM E₂. mRNA levels of *hPYGO2* and Cathepsin D (*CTSD*), a well-established E₂ responsive gene (Shang et al., 2000), were measured after 0.5, 1, 4, 8 and 24 h by Q-PCR (Figure 3.5 A, C, E). *hPYGO2* mRNA expression was slightly higher (2.1-3.9 fold, respectively) after 30 m of E₂ treatment in the ER α + cell lines, relative to cells treated with the Et control (which was set to one) for the same time point. *hPYGO2* mRNA levels peaked (9.3 fold, $p < 0.01$, in MCF7 and 11.9 fold, $p = 0.012$, in MC2) after 4 h and were still higher than Et treated cells after 1 d. The expression of *CTSD* followed the same trend but exhibited a higher overall induction (5.1-7.6 fold after 30 m, 18-21.9 fold at the 4 h point and 2.2-9.2 fold after 1 d), which is consistent with previous observations (Levenson and Jordan, 1994; Krishnan et al., 1994; Shang et al., 2000).

Figure 3.5 Expression analysis of hPygopus2 in ER α + and ER α - BrCa cells lines after E₂ exposure.

Expression levels of *hPYGO2* and *CTSD* mRNA as analyzed by Q-PCR in MCF7 (A), VC5 (C) and MC2 (E) cell lines after treatment with either Et or E₂ for indicated time points. mRNA levels were normalized to β -Actin and set to 1 in the 30 m Et treated samples and adjusted accordingly for other time points. Expression levels of hPYGO2, CTSD and ER α protein as measured by immunoblot in MCF7 (B), VC5 (D) and MC2 (F) cell lines after treatment with either Et or E₂ for indicated time points. β -Actin was used as a loading control and molecular weight is expressed in kDa. (G) Expression levels of *hPYGO2* mRNA as analyzed by Q-PCR in MDA-MB-231 cells after mock, empty vector or ER α expression vector transfection and treatment with either Et or E₂ for 1 h. mRNA levels were normalized to β -Actin and set to 1 in the mock Et treated sample and adjusted accordingly for other time points. (H) Expression levels of hPYGO2 protein was measured by immunoblot in MDA-MB-231 cells after mock, empty vector or ER α expression vector transfection and treatment with either Et or E₂ for 6 h. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=). β -Actin was used as a loading control and molecular weight is expressed in kDa.



Changes in hPYGO2 protein levels were assessed after 1, 4, 8, and 24 h of E₂ treatment by immunoblot (Figure 3.5 B, D, F). In MCF7 cells, there was a 4 fold increase after 8 h (p=0.036) and a 6 fold increase after 1 d (p=0.021). In MC2 cells, there was a noticeable increase after 4 h (p=0.043), which was maintained after 8 h (p=0.041) and peaked after 1 d (p=0.025). Induction of CTSD protein followed a similar trend in both these cell lines. I specifically focused on the levels of preprocathepsin D because this is the only product transcribed and the first product to be translated.

There was no significant change in hPygopus2 and Cathespin D mRNA and protein levels after E₂ treatment in VC5 cells, although some minor variability was observed. Expression of ER α in the appropriate cells lines was confirmed by immunoblot and there was also no change in its expression after any treatment as expected (Shang et al., 2000).

MDA-MB-231 cells transiently transfected with ER α for 1 d and treated with 100 nM E₂ for 1 and 6 h displayed similar inductions of hPygopus2 mRNA and protein, respectively (Figure 3.5 G and H). These results suggested that hPygopus2 mRNA and protein levels are elevated by 17 β -estradiol in ER α + BrCa cells.

3.3.3 17 β -estradiol induction of hPygopus2 does not require the E74-like factor 1

The correlation between ER α and hPYGO2 expression coupled with the 17 β -estradiol mediated enhancement of hPygopus2 led me to examine the mechanism of ER α -mediated hPygopus2 expression. As we have previously shown the ELF1

transcription factor is able to induce hPygopus2 expression (Andrews et al., 2008), I examined if E₂ induction of *hPYGO2* required ELF1. E₂ could potentially indirectly induce *hPYGO2* through ELF1 by transcribing *CCND*, the translated product of which would complex with CDK4/6 to phosphorylate RB and release ELF1.

To establish that ELF1 (specifically) is not required for E₂-mediated induction of *hPYGO2*, I treated cells with E₂ after either ELF1 knockdown by RNAi or ELF1 overexpression by transient transfection. hPYGO2 and ELF1 protein expression was measured by immunoblot.

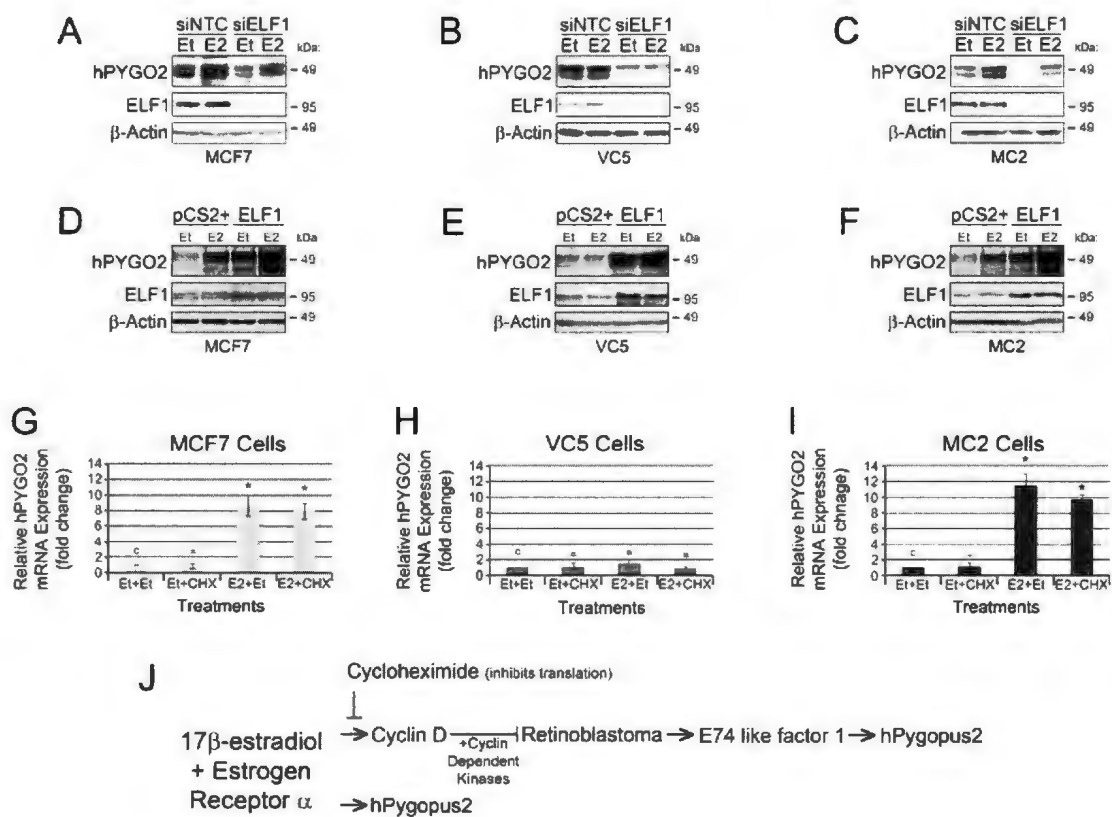
siELF1 effectively reduced ELF1 and hPYGO2 protein levels in MCF7, VC5 and MC2 cells lines (Figure 3.6 A, B, C). In contrast, E₂ treatment after ELF1 knockdown enhanced hPYGO2 expression in MCF7 and MC2 cell lines while having no effect on hPYGO2 expression in VC5 cells, relative to the Et control.

Overexpression of ELF1 caused an obvious increase in both ELF1 and hPYGO2 expression levels (Figure 3.6 D, E, F). E₂ treatment resulted in increased hPYGO2 levels relative to the Et control in both empty vector and ELF1 overexpressed samples in MCF7 and MC2 cell lines. ELF1 overexpression increased hPYGO2 protein levels in VC5 cells but was not further increased by E₂ treatment.

To assess if E₂ induces *hPYGO2* directly, I treated BrCa cells with the protein synthesis inhibitor cyclohexamide (CHX) prior to adding E₂. CHX treatment prevents the translation of any potential intermediate proteins thereby allowing me to determine if hPygopus2 expression is an immediate early response to E₂.

Figure 3.6 The requirement of ELF1 in E₂-mediated enhancement of hPygopus2 in ER α + and ER α - BrCa cell lines.

Expression levels of hPYGO2 and ELF1 protein as measured by immunoblot after treatment with Et or E₂ and treatment with ELF1 directed siRNA or a non-target control siRNA in MCF7 (A), VC5 (B) and MC2 (C) cell lines. Expression levels of hPYGO2 and ELF1 protein as measured by immunoblot after treatment with Et or E₂ and overexpression of ELF1 or an empty vector control in MCF7 (D), VC5 (E) and MC2 (F) cell lines. β -Actin was used as a loading control for the immunoblots and molecular weight is expressed in kDa. Expression levels of *hPYGO2* mRNA as analyzed by Q-PCR after 1 h of Et or E₂ treatment after pretreatment with cyclohexamide (CHX) for 2 h in MCF7 (G), VC5 (H) and MC2 (I) cell lines. mRNA levels were normalized to *β -Actin* and set to 1 in the Et-Et treated sample and adjusted accordingly for the other samples. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=). (J) Schematic displaying potential pathways by which E₂-ER α can indirectly and directly induce hPygopus2 expression.



The effects of E₂ after CHX treatment were assessed by Q-PCR using MCF7, VC5 and MC2 BrCa cells (Figure 3.6 G, H, I). Pretreatment with CHX for 2 h did not significantly alter *hPYGO2* mRNA levels relative to the Et control in any of the cell lines. In MCF7 and MC2 cells, 1 h of E₂ treatment increased *hPYGO2* mRNA by 8.7 fold (p<0.01) and 11.54 fold (p<0.01), respectively, relative to the control and CHX pretreatment did not prevent this increase (8 and 9.7 fold, respectively, p<0.01 for both). E₂ treatment had no effect on *hPYGO2* levels in VC5 cells. The induction of *hPYGO2* by E₂ in ERα+ cells, despite pretreatment with CHX, suggested that *hPYGO2* may be a direct E₂ target gene in these cells.

The foregoing results demonstrating E₂ induction of hPYGO2 protein without the requirement of translation of an intermediary product or the requirement for ELF1 in E₂ responsive cell lines suggested that E₂ induction of *hPYGO2* occurred by a mechanism independent of ELF1, potentially involving ERα (Figure 3.6 J).

3.3.4 Four-hydroxytamoxifen and fulvestrant prevent 17β-estradiol mediated induction of hPygopus2

As ERα, is the best characterized mechanism by which E₂ induces gene expression, we examined the requirement of ERα in E₂-mediated *hPYGO2* induction. To do this I utilized two ERα antagonists (4-OHT and FUL), both of which compete with E₂ for occupation of the ligand binding site on ERα (FUL additionally induces ERα

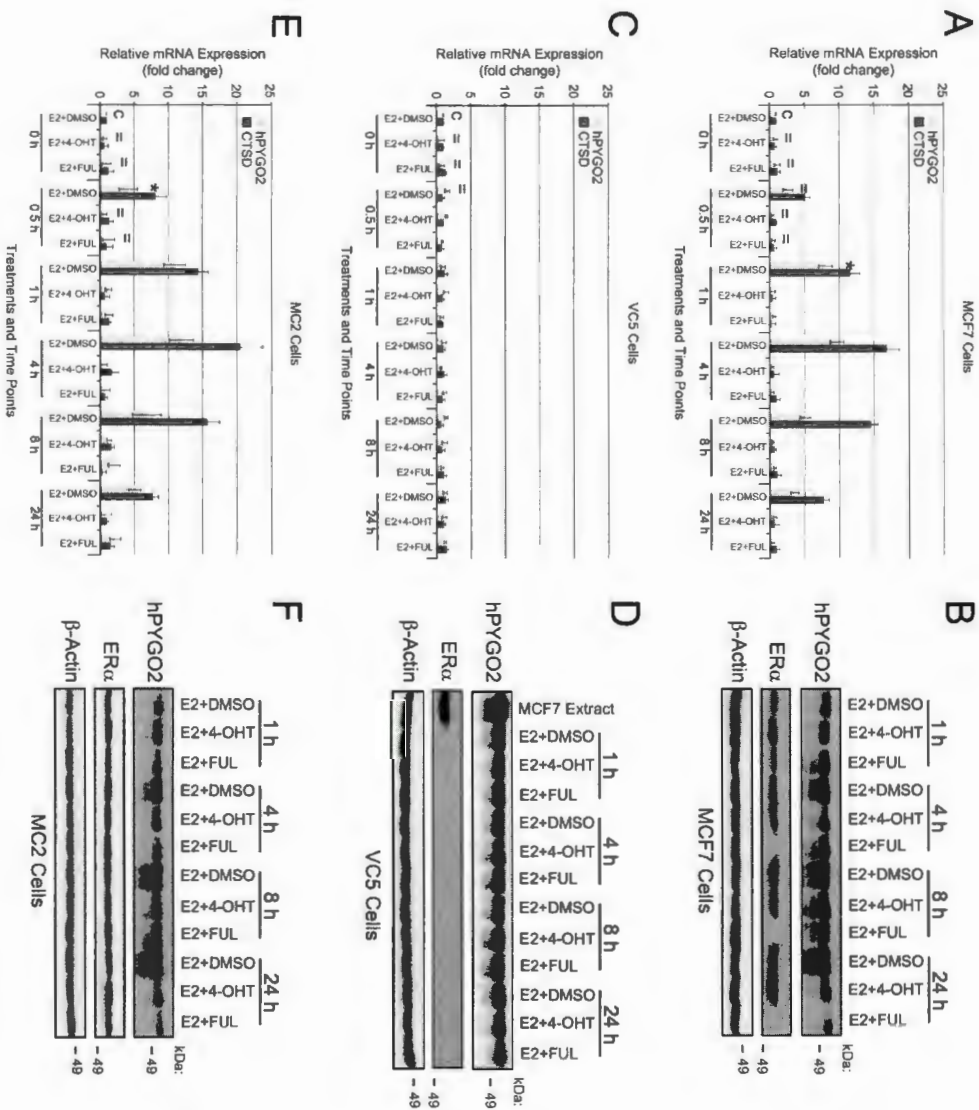
degradation) and thereby prevent E₂ directed gene activation through ER α . Thus, I assessed the ability of 4-OHT and FUL to prevent E₂-mediated induction of *hPYGO2*.

MCF7, MC2 and VC5 cells were hormone deprived for 3 d and then pretreated with DMSO, 4-OHT and FUL for 6 h. E₂ was added to all samples at the 6 h mark and *hPYGO2* and *CTSD* mRNA levels (as measured by Q-PCR) were measured at 0 (before E₂ treatment, control), 0.5, 1, 4, 8 and 24 h. In the control sample (0 h), 4-OHT and FUL treatment did not significantly change *hPYGO2* and *CTSD* mRNA levels relative to the DMSO pretreatment (which was set to one) (Figure 3.7 A, C, E). For the remainder of the time points, E₂ induction of *hPYGO2* and *CTSD* mRNA in the DMSO pretreated samples was similar to the E₂ induction in MCF7 and MC2 cells observed earlier (Figure 3.5 A and C). However, pretreatment with either 4-OHT or FUL effectively prevented E₂-mediated induction of both *hPYGO2* ($p>0.05$) and *CTSD* mRNA, maintaining their levels to that of the control (DMSO, time 0). This was observed in both ER α + MCF7 and MC2 BrCa cell lines. This level of *CTSD* induction in these experiments is consistent with observed in previous studies (Levenson and Jordan, 1994 and Krishnan et al., 1994).

The effects of 4-OHT and FUL pretreatment on hPYGO2 protein levels were also evaluated 1, 4, 8, and 24 h after the addition of E₂ by immunoblot. In the 1 h control sample, the hPYGO2 protein levels were unaffected by any of the treatments (Figure 3.7 B, D, F). The induced expression of hPYGO2 protein by E₂ in the DMSO pretreated samples was similar to the E₂ induction observed earlier (Figure 3.5 B and F). In MCF7 cells, the E₂-mediated induction at 4 and 8 h was effectively inhibited by 4-OHT and

Figure 3.7 Expression analysis of hPygopus2 in ER α + and ER α - BrCa cells lines treated with E₂ after pretreatment with 4-OHT and FUL.

Expression levels of *hPYGO2* and *CTSD* mRNA as analyzed by Q-PCR in MCF7 (A), VC5 (C) and MC2 (E) cell lines after treatment with either Et, or E₂ after pretreatment with DMSO, 4-OHT or FUL for indicated time points. mRNA levels were normalized to β -Actin and set to 1 in the 30 m Et and DMSO treated samples and adjusted accordingly for other time points. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=). Expression levels of hPYGO2, CTSD and ER α protein as measured by immunoblot in MCF7 (B), VC5 (B) and MC2 (F) cell lines after treatment with either Et or E₂ after pretreatment with DMSO, 4-OHT or FUL for indicated time points. β -Actin was used as a loading control and molecular weight is expressed in kDa.



FUL pretreatment. There was actually a slight decrease in hPYGO2 protein levels in the 1 d treated sample. A consistent trend of induction was observed with MC2 cells.

As a positive control, I measured the protein levels of ER α . Consistent with previous results (Pink and Jordan, 1996), E₂ and 4-OHT treatment did not significantly alter the level of ER α . However, as expected (Howell, 2006), FUL treatment resulted in lowered ER α levels at the 1 and 4 h time points and ER α was completely absent after 8 h. In the MC2 line there was no significant reduction of ER α protein levels, presumably due to its constitutive expression, but there was a reduction of ER α translocation to the nucleus (appendix Figure 6.2), as expected (Howell, 2006).

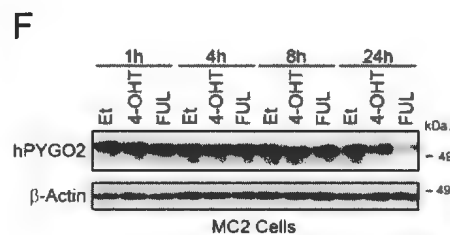
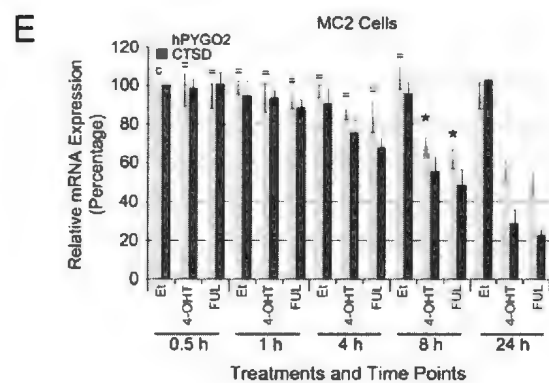
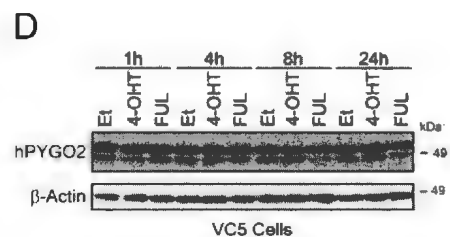
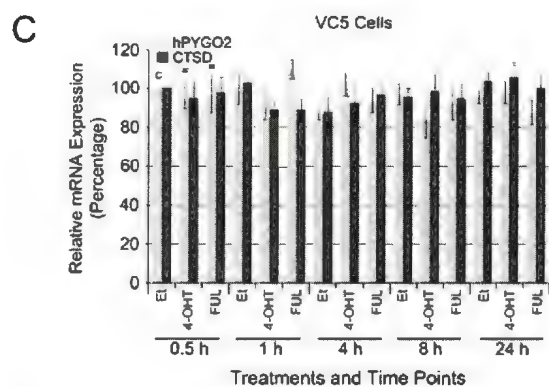
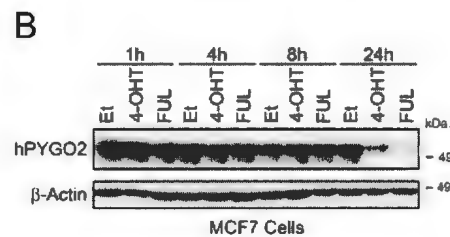
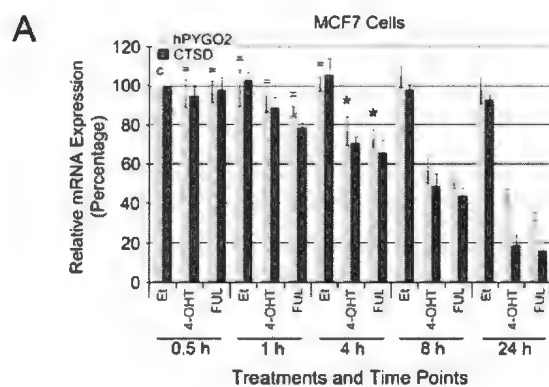
Consistent with the lack of ER α in the VC5 cell line, neither the 4-OHT or FUL pretreatment nor the E₂ treatment had any effect on hPygopus2 mRNA or protein levels in these cells. Together, these results suggest that ER α is required for the E₂-mediated induction of hPygopus2.

3.3.5 *Four-hydroxytamoxifen and fulvestrant reduce hPygopus2 levels*

The requirement of ER α for E₂ induced hPygopus2 expression was tested using an alternate method. MCF7, MC2 and VC5 cells were grown in low serum (1% FBS) conditions and treated with Et (control, which was set to 100% for the 30 m time point), 4-OHT or FUL. hPygopus2 mRNA (as measured by Q-PCR) and protein (as measured by immunoblot) levels were assessed at various time points (Figure 3.8 A, C, E and B, D, F, respectively). Significant reductions in hPYGO2 mRNA levels after 4-OHT

Figure 3.8 Expression analysis of hPygopus2 in ER α + and ER α - BrCa cells lines treated with 4-OHT and FUL.

Expression levels of *hPYGO2* and *CTSD* mRNA as analyzed by Q-PCR in MCF7 (A), VC5 (C) and MC2 (E) cell lines after Et, 4-OHT or FUL treatment for indicated time points. mRNA levels were normalized to β -Actin and set to 1 in the 30 m Et treated samples and adjusted accordingly for other time points. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=). Expression levels of hPYGO2 protein as measured by immunoblot in MCF7 (B), VC5 (B) and MC2 (F) cell lines after treatment with Et, 4-OHT or FUL for indicated time points. β -Actin was used as a loading control and molecular weight is expressed in kDa.



and FUL treatments were observed after 4 h for MCF7 cells and after 8 h for MC2 cells (less than 58%, $p < 0.01$, for 4-OHT and less than 53%, $p < 0.01$, for FUL) relative to the control samples at these time points.

A significant reduction in hPYGO2 protein levels, relative to the control, was not observed until the 1 d time point after 4-OHT or FUL treatment in the ER α + cell lines. This may have been due to the long half-life of hPYGO2 (approximately 40 h, data not shown). While there was some variability in hPygopus2 mRNA and protein levels in the VC5 cells, there was neither a consistent nor a significant reduction in response to 4-OHT and FUL. These findings suggested that E₂ induction of *hPYGO2* is mediated by ER α .

3.3.6 The Estrogen receptor alpha and SP1 transcription factor bind to the hPygopus2 promoter

The requirement of ER α in the 17 β -estradiol directed induction of *hPYGO2* and the relatively rapid rate of its transcription suggested that ER induction of *hPYGO2* occurs through one of two ways. These include ER α stimulation via activation of kinases/phosphatases, which activate downstream transcription factors, or more directly by the binding of ER α to the *hPYGO2* gene promoter. To initially rule out or include ER α as a promoter-binding candidate, I performed an *in silico* analysis of a 1494 bp segment of *hPYGO2* promoter using Transcription Element Search System software. Both direct and indirect ER α binding was considered by paying particular attention to ERE palindromes, ERE half-sites, GC-boxes (SP1 binding sites), AP-1 and NF κ B

binding sites. While there were no putative AP-1 or NF κ B binding sites, however, there were six ERE half-sites and twenty GC-boxes (Figure 3.9 A).

Due to the uniform distribution of these sites, I decided to narrow the region by using several previously generated luciferase reporter constructs (Andrews et al., 2008). The largest of these constructs contains the 1494 bp region upstream of the *hPYGO2* transcription start site (pGL3-1494) and the rest are progressively smaller by approximately 300 bp at a time (pGL3-1143, -829, -531 and -225) (Figure 3.9 A). The negative control was a reporter vector that did not contain any promoter DNA (pGL3-basic). I hypothesized that overexpression of these reporter constructs followed by E₂ stimulation would identify the E₂ responsive/sensitive region(s) within the *hPYGO2* promoter.

In order to induce any quantifiable E₂-mediated promoter activity from a reporter construct, ER α must be coexpressed (Miralles et al., 1994). To be thorough, I coexpressed different combinations of expression vectors with each reporter construct. These combinations included an empty vector control, ER α and SP1 alone and ER α and SP1 together.

When the reporter constructs were coexpressed with each combination of expression vectors in MCF7 cells, promoter activity was observed for two reporter constructs relative to the empty vector control, pGL3-1494 and pGL3-531 (Figure 3.9 B). In both cases, promoter activity was slightly increased (less than 2.1 fold) by overexpression of SP1 or ER α alone, relative to the empty vector control. The highest promoter activity was obtained when both ER α and SP1 were coexpressed (4.2 fold,

$p=0.032$) and increased even more when supplemented with E_2 (10.2 fold, $p<0.01$, for pGL3-1494 and 4.3 fold, $p=0.035$, for pGL3-531). The level of luciferase activity observed here is within the range observed in previous studies (Krishnan et al., 1994).

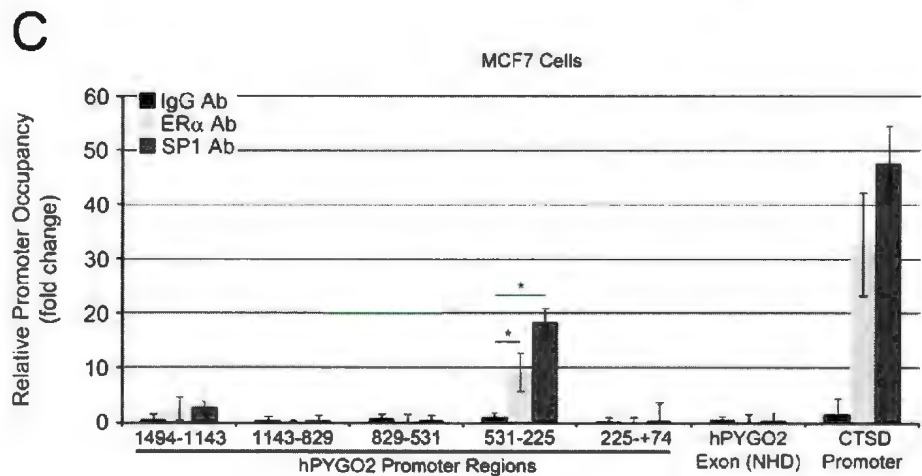
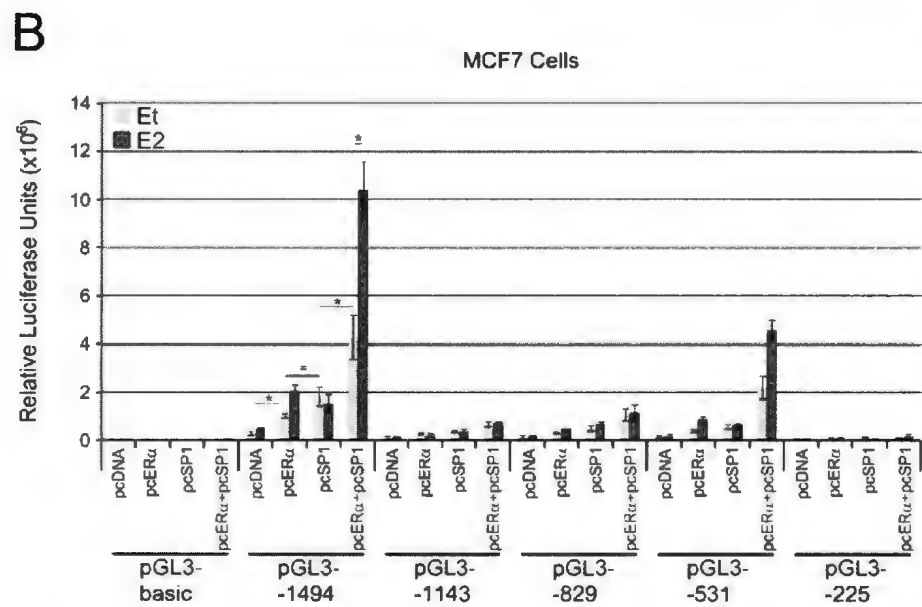
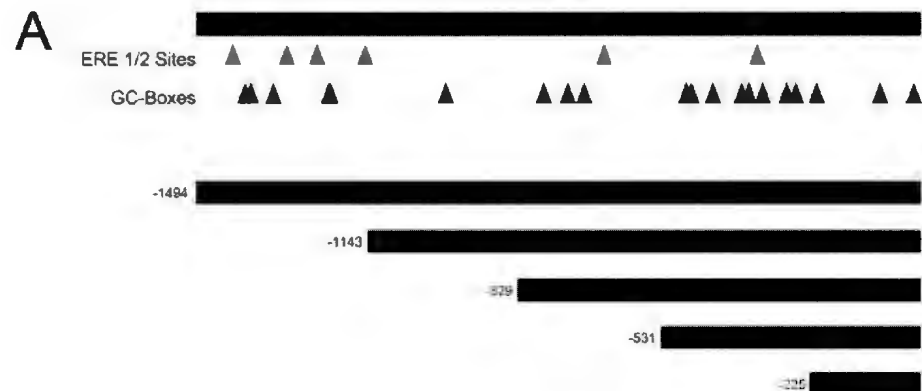
While this experiment excluded the E_2 non-responsive regions, it did not distinguish between the two responsive regions. Furthermore, while it did not discriminate between rapid effects or direct ER binding, but it did highlight the importance of SP1 in addition to $ER\alpha$.

The synergistic induction of *hPYGO2* promoter activity when $ER\alpha$ and SP1 were coexpressed, especially in the presence of E_2 , supported the idea that $ER\alpha$ may bind to the *hPYGO2* promoter in a complex with SP1. I performed ChIP assays to determine the presence of $ER\alpha$ and SP1 at the *hPYGO2* promoter. Hormone deprived MCF7 cells were treated with E_2 and processed for ChIP analysis after 45 m (established as time point during which ER displays maximal binding) (Shang et al., 2000). Protein complex cross-linked DNA samples were immunoprecipitated with Immunoglobulin G (IgG, control antibody), $ER\alpha$ or SP1 antibodies. DNA isolated from the complexes were subjected to Q-PCR to test for amplification of each of the five regions within 1494 bp promoter DNA. The IgG control for the -531 to -225 segment was set to 1 for comparative purposes.

Both $ER\alpha$ and SP1 displayed promoter occupancy of the -531 to -225 region in E_2 treated MCF7 cells (Figure 3.9 C). $ER\alpha$ and SP1 bound 9.3 fold ($p=0.01$) and 18.5 fold ($p<0.01$) higher, respectively, than the IgG control for this region. As a negative control, I

Figure 3.9 ER α and SP1 binding to the *hPYGO2* promoter in MCF7 cells.

(A) Schematic displaying ERE half-sites and GC-boxes in *hPYGO2* promoter and the relative lengths of the progressive luciferase constructs. (B) Relative induction of each *hPYGO2* promoter construct after transfection of ER α and SP1 expression vectors and Et or E₂ treatment for 1 d. Luciferase activity was normalized to β -gal activity and displayed as fold change. (C) ChIP assays measuring ER α and SP1 occupancy of different *hPYGO2* promoter regions after E₂ treatment for 1 h. Promoter occupancy was analyzed by Q-PCR and normalized to IgG in the -1494 to -1143 region and the rest were adjusted accordingly. ER α and SP1 binding to *hPYGO2* exonic region and *CTSD* promoter was also analyzed. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample, are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=).



confirmed that ER α and SP1 binding to a *hPYGO2* exonic region was not significantly higher than the IgG control. As a positive control, I confirmed that the strength of ER α and SP1 binding to a previously established ER α -SP1 target gene, *CTSD*, (Castro-Rivera et al., 2001) was within the expected range.

These findings suggested that 17 β -estradiol stimulation of hPygopus2 expression occurs as a result of ER α and SP1 occupancy of the *hPYGO2* promoter within the -531 to -225 region. These results are consistent with the region identified by the luciferase assay (Figure 3.9 B)

3.3.7 An estrogen response element half-site and a GC-box are required for Estrogen receptor alpha and SP1 transcription factor binding

The binding of ER α and SP1 to the -531 to -225 segment of the *hPYGO2* promoter clearly highlighted their importance in 17 β -estradiol mediated induction of *hPYGO2*. However, there is one ERE half-site and eight GC-boxes within this region. Considering that ER α can bind directly or be indirectly tethered to DNA through SP1, any of these sites may be responsible for mediating the interaction.

To determine which sites are required for ER α and SP1 binding to the *hPYGO2* promoter, I changed the sequence of the only ERE half-site (at the -331 bp location) and the highest predicted GC-box (at the -356 bp location) by site-directed mutagenesis. These mutations, which have been shown to render these sites non-functional (Krishnan et al., 1994), were made in three combinations in the pGL3-531 reporter construct (mut-

ERE, mut-GC, mut-ERE+GC) (Figure 3.10 A). The negative control was the pGL3-basic reporter construct (no promoter sequence) and the positive control was the wildtype pGL3-531 reporter construct.

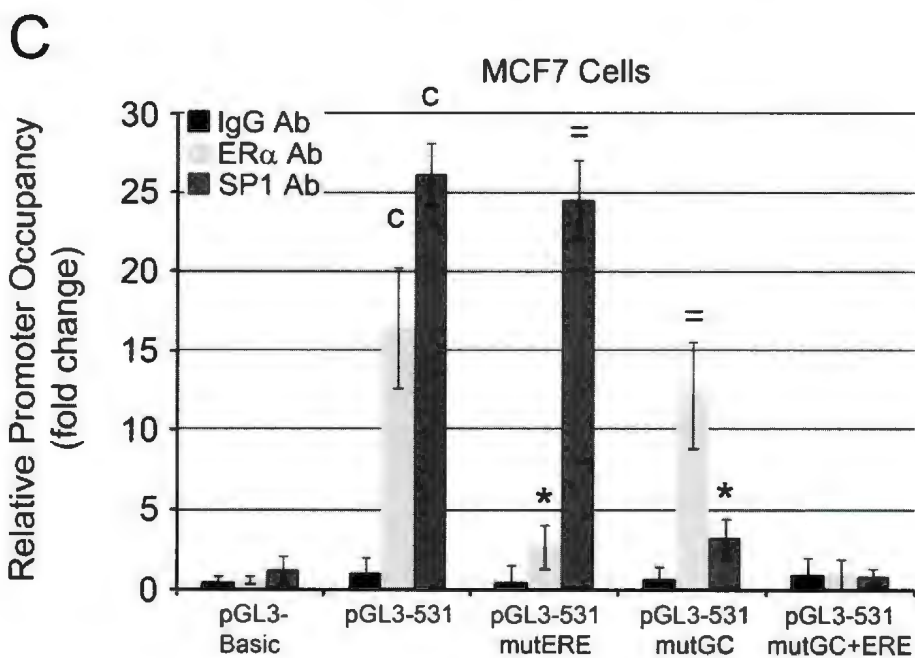
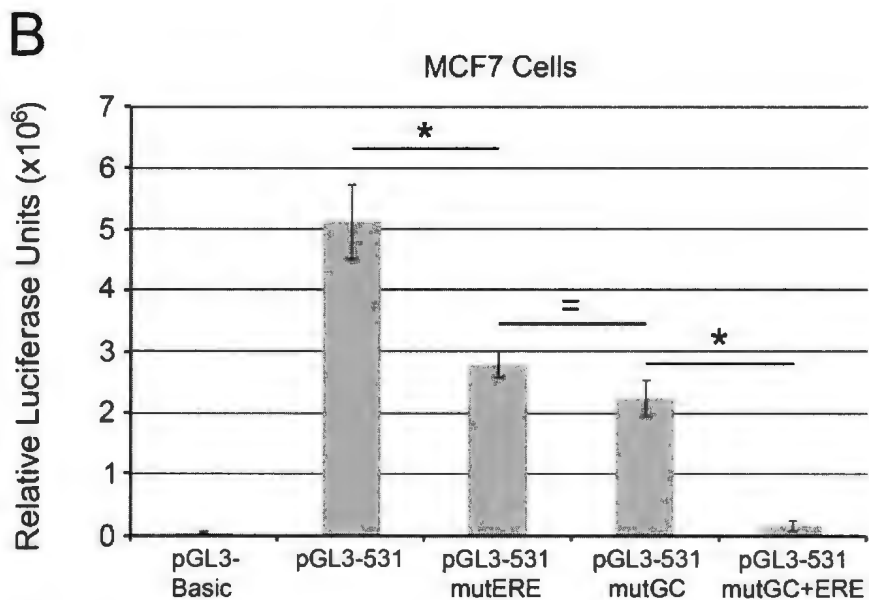
MCF7 cells deprived of hormones were co-transfected with both ER α and SP1 expression vectors and each of the reporter constructs and then treated with E₂ for 1 d. Promoter activity of the wildtype pGL3-531 reporter construct was similar to that reported earlier (Figure 3.9 B). Mutation of either the ERE half-site or GC-boxes caused comparable and significant reductions in promoter activity. The pGL3-531 mut-ERE construct promoter activity was reduced to just over half (54.5%, $p < 0.01$), while the pGL3-531 mut-GC construct was reduced to just under half (43.8%, $p < 0.01$), relative to the wildtype vector (Figure 3.10 B). Interestingly, the resulting reporter activity of the pGL3-531 mut-ERE+GC construct was not significantly different from the empty vector control (pGL3-basic).

These results suggested that both the ERE half-site (at -331) and the GC-box (at -356) are required for maximal activity of the -531 to -225 segment of *hPYGO2* promoter and that the contribution of each is equal, but not dependent on the other. Additionally, the observation that rendering both sites non-functional removes all E₂ responsiveness of this region suggested that no other factors (besides ER α and SP1) are involved in the E₂ directed activation of this *hPYGO2* promoter region. This thereby excludes the roles of factors that would be activated through E₂-mediated nongenomic signalling.

Figure 3.10 Identification of the required ERE half-site and GC-box in the *hPYGO2* promoter in MCF7 cells.

(A) Sequence of putative required ERE half-site and GC-box in *hPYGO2* promoter and site directed mutated bases. (B) Relative induction of wildtype and mutated *hPYGO2* pGL3-531 reporter constructs after transfection of ER α and SP1 expression vectors and 1 d of E₂ treatment. Luciferase activity was normalized to β -gal activity and displayed as relative luciferase units. (B) ChIP assays measuring transfected ER α and SP1 occupancy to the pGL3-531 wildtype and mutated *hPYGO2* promoter reporter vector after E₂ treatment. Promoter occupancy was analyzed by Q-PCR and normalized to IgG in the pGL3-531 basic region and the remaining samples were adjusted accordingly. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=).

A	Vector Name	GC (-356)-ERE1/2 Site (-341) Mutations
	pGL3-531	CCGCCC-n9-TGCCC
	pGL3-531mutGC	CTGTCC-n9-TGCCC
	pGL3-531mutERE	CCGCCC-n9-TATAG
	pGL3-531mutGC+ERE	CTGTCC-n9-TATAG



The requirement of both the ERE half-site and the GC-box for maximal promoter activity and their equal contribution suggested that not only do both ER α and SP1 bind directly to these DNA sites, but that their binding is independent of one another.

To assess the requirement of the ERE half-site and the GC-box for the binding of ER α and SP1, respectively, and to determine if the binding of one factor is independent of the other, I performed ChIP assays using the site-mutated pGL3-531 reporter constructs. Hormone deprived MCF7 cells were cotransfected with one of the pGL3-531 mutated reporters (mut-ERE, mut-GC, mut-ERE+GC) and the expression vectors (pcERa, pcSP1 or both), treated with E₂, processed for ChIP analysis and immunoprecipitated with IgG, ER α and SP1 antibodies. Specific regions of the isolated DNA were amplified and measured by Q-PCR. The negative and positive controls were the pGL3-basic and pGL3-531 reporter constructs, respectively.

Binding of ER α and SP1 to the pGL3-531 was easily detectable at 16.4 fold ($p=0.018$) and 26.1 fold ($p<0.01$), respectively, above the IgG control (Figure 3.10 C). Mutation of the ERE-half site in the *hPYGO2* promoter resulted in a 6.3 fold ($p=0.033$) reduction in ER α promoter occupancy relative to occupancy in the wildtype vector, rendering it not significantly different from the IgG control. The mutation in the pGL3-mut-ERE reporter construct did not affect SP1 binding. Site-directed mutagenesis of the GC-box caused an 8.1 fold reduction ($p=0.026$) in SP1 binding relative to that in the wildtype construct and made it non-significantly different from the IgG control. ER α binding to the pGL3-mut-GC reporter construct, although slightly reduced, was not significantly different than ER α binding to the pGL3-531 reporter construct. However,

when both the ERE half-site and the GC-box were rendered non-functional, the binding either of ER α or SP1 was not significantly higher than the IgG control.

These findings suggested that not only do both ER α and SP1 bind directly to the -531 to -225 region of the *hPYGO2* promoter but also that their binding is specifically and solely mediated by the ERE half-site at -331 and the GC-box at -356. Furthermore, these results suggested that binding of ER α is independent of SP1 and vice versa.

3.3.8 Inhibition of Estrogen receptor alpha does not prevent SP1 transcription factor binding to the hPygopus2 promoter and vice versa

The foregoing results suggested that ER α and SP1 binding to the *hPYGO2* promoter is not dependent on the binding of the other factor. The specific mechanism of ER α and SP1 binding (direct or tethering) to a target gene promoter is highly debated and difficult to decipher. To gain a better understanding of ER α and SP1 dynamics and requirements at the *hPYGO2* promoter, I specifically targeted each factor and subsequently assessed how its promoter binding, and the promoter binding of its partner, was affected.

ER α agonists were used to activate ER α and induce promoter binding while pretreatment with antagonists was used to inactivate and block the effects of E₂. MCF7 cells were pretreated with DMSO, 4-OHT or FUL following growth in hormone free media for 3 d. After the 6 h, DMSO pretreated cells were treated with Et or E₂ and 4-OHT and FUL pretreated cells were treated with E₂. Cell samples were collected after

various time points, processed for ChIP analysis and the binding of IgG, ER α and SP1 was quantified on both the *hPYGO2* and *CTSD* promoters by Q-PCR.

The association of ER α with the *hPYGO2* promoter was unaffected by Et (control) and was not significantly different from that of IgG (Figure 3.11 A). E₂ treatment resulted in a significant increase (3.2 fold, $p=0.029$) in ER α binding to the *hPYGO2* promoter after 15 m, relative to the Et control at the same time point. This increase peaked (11.4 fold) at 30 m and was not significantly different after 1 or 2 h. As expected, pretreatment with 4-OHT resulted in virtually the same induction pattern (Shang et al., 2000). Relative to the Et control samples, ER α binding increased (2.5 fold) after 15 m, was highest at 30 m (10.4 fold, $p<0.01$) and not significantly different after 1 and 2 h. As expected (Shang et al., 2000), pretreatment with FUL caused a slight not significant increase (1.3 to 2.1 fold) in ER α binding to the *hPYGO2* promoter after 15 and 30 m and 2 h. The increase after 1 h was significant (2.6 fold, $p=0.042$) but was much lower than the E₂ and 4-OHT-mediated ER α binding at this time point.

ER α binding to the promoter of the positive control gene, *CTSD*, followed a similar pattern to ER α binding to the *hPYGO2* promoter, except the overall level of binding relative to the Et control was higher (Figure 3.11 A). Et treatment had no effect on ER α occupancy of the *CTSD* promoter relative to IgG levels. E₂ treatment and 4-OHT pretreatment induced ER α promoter binding with a significant increase (5.3 fold) after 15 m and a peak (20.7 fold) was observed after 1 h in response to both treatments, in relation to the Et treatments at these time points. Pretreatment with FUL also caused a slight non-significant increase after 15 m and the highest level of binding (2.4 fold), which was

significant, occurred at 30 min and 1 h (3.8 and 4.6 fold, respectively). These results are consistent with previous findings (Shang et al., 2000)

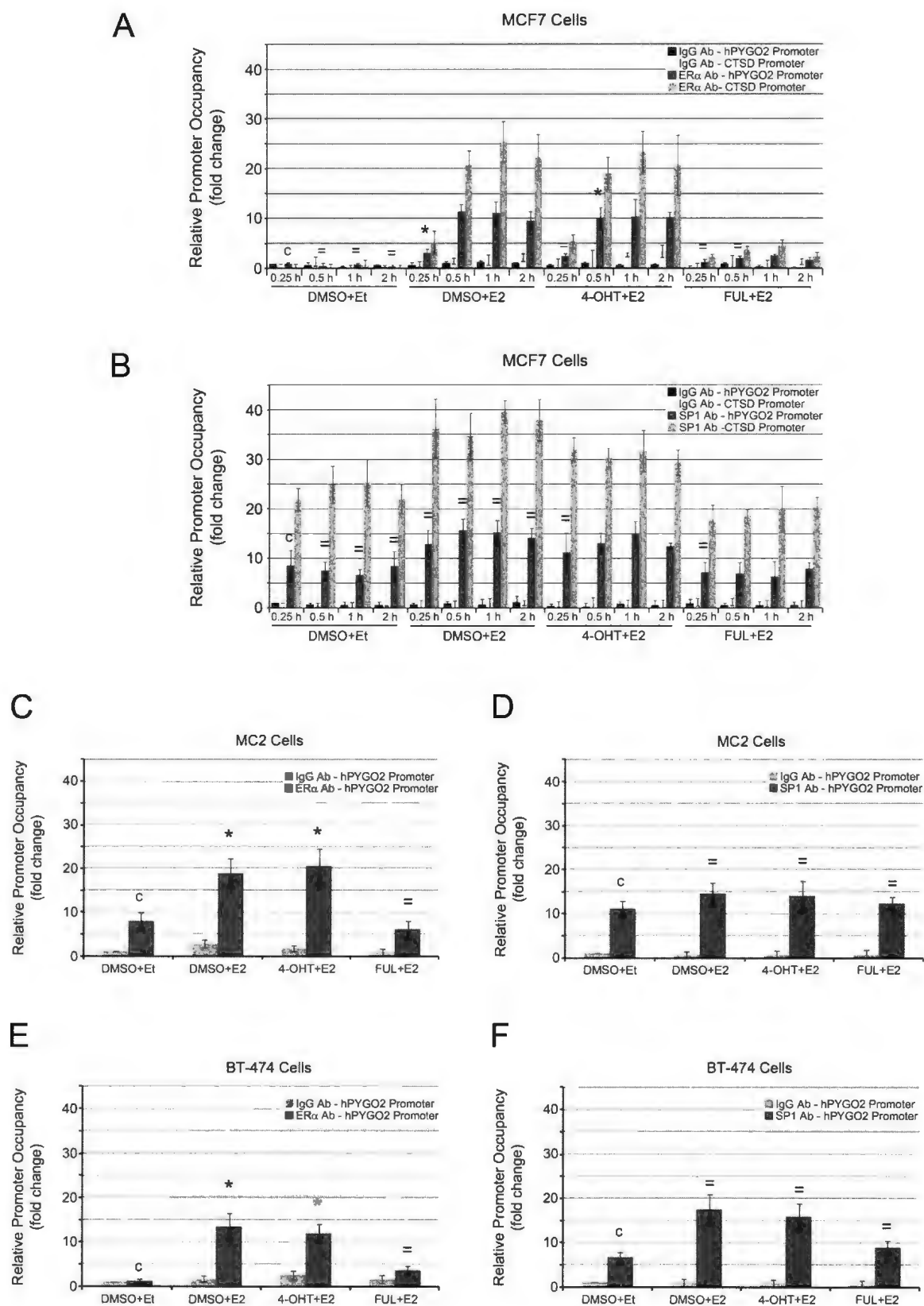
The effect of ER α stimulatory and inhibitory ligands on SP1 occupancy of the *hPYGO2* and *CTSD* promoters was also assessed (Figure 3.11 B). Interestingly, SP1 binding to *hPYGO2* promoter in the Et control was 8.6 fold ($p < 0.01$) higher than the IgG control and not significantly different between time points. Thus, SP1 occupied this promoter even in hormone free conditions. Treatment with E₂ and pretreatment with 4-OHT caused a slight increase (between 11.2 and 15.6 fold) in SP1 promoter occupancy, relative to the Et controls, but this increase was not significant for any of the time points. SP1 promoter binding after FUL pretreatment did not vary from the Et treated samples across all time points, but was lower than the E₂ and 4-OHT samples.

The binding of SP1 to the *CTSD* promoter after agonist and antagonist treatment, caused the same pattern of binding as to the *hPYGO2* promoter, except that SP1 binding to the *CTSD* promoter was stronger (Figure 3.11 B). SP1 promoter occupancy was significantly higher (between 21.9 and 25.3 fold) than the IgG control in the Et treated samples. Both the E₂ treatment and the 4-OHT pretreatment caused an increase (between 34.7 and 39.6 fold and between 29.3 and 31.7 fold, respectively) in SP1 promoter occupancy, but the only significant increases were for the E₂ treated samples (all time points). Pretreatment with FUL did not significantly affect SP1 occupancy of the *CTSD* promoter relative to the Et treated samples.

Consistent results demonstrating increased ER α binding to the *hPYGO2* promoter after E₂ and 4-OHT treatment and reduced ER α binding after FUL treatment were also

Figure 3.11 Determination of the ER α requirement for SP1 binding to the *hPYGO2* promoter in ER α + BrCa cell lines.

ChIP assays measuring ER α (A) and SP1 (B) occupancy to the *hPYGO2* and *CTSD* promoter after treatment with E₂, 4-OHT and/or FUL at different time points in MCF7 cells. Binding of ER α and SP1 to the *hPYGO2* promoter in MC2 (C and D, respectively) and BT-474 (E and F, respectively) after 1 h of treatment with E₂, 4-OHT and/or FUL. In all cases promoter occupancy was analyzed by Q-PCR and normalized to IgG in the DMSO+Et sample and adjusted accordingly in the remainder of the samples. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=).



obtained in MC2 and BT-474 cell lines (Figure 3.11 C and E). Additionally, no significant change in SP1 binding to the *hPYGO2* promoter was observed after E₂, 4-OHT and FUL treatments in MC2 and BT-474 cell lines (Figure 3.11 D and F).

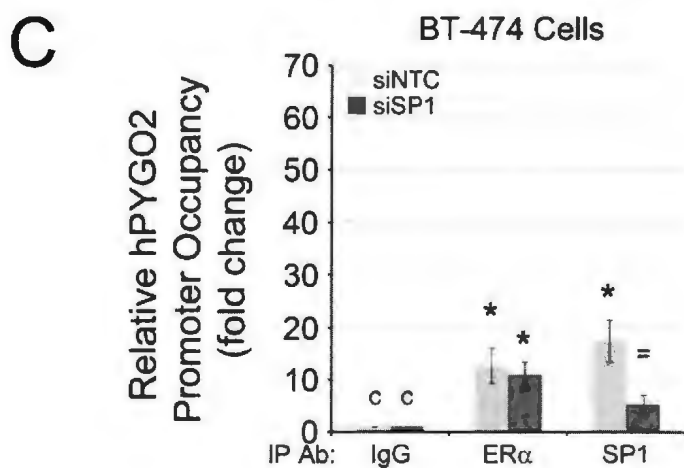
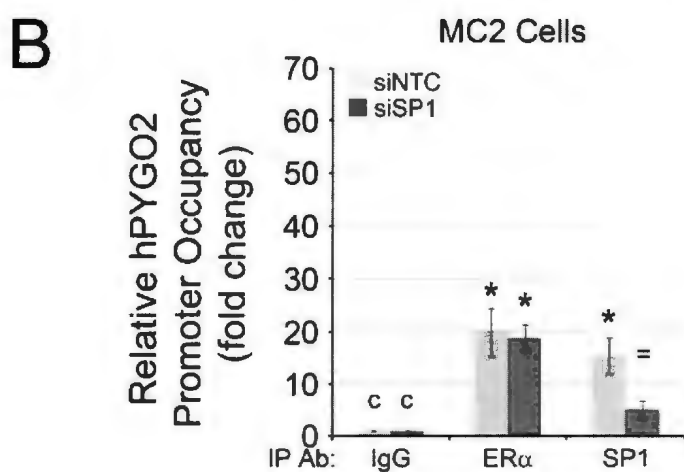
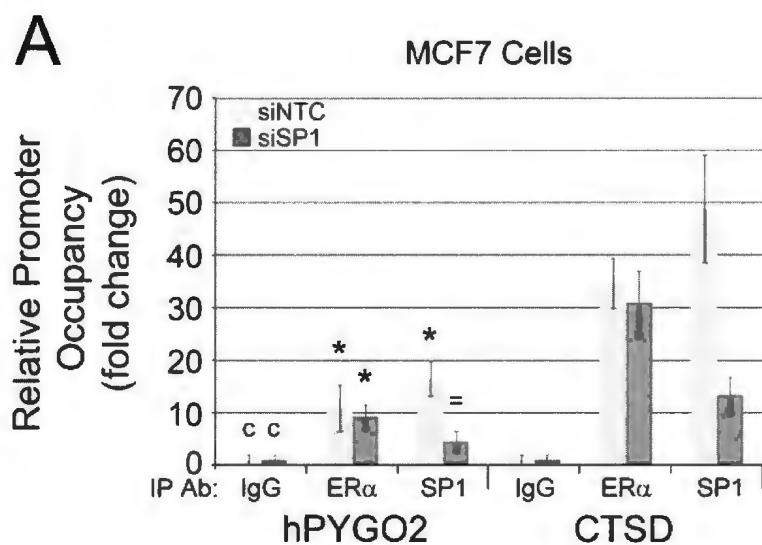
These results suggested that treatment with different ER α ligands significantly altered the binding of ER α to the *hPYGO2* promoter. However, the effects that these ligands exhibited on SP1 binding to the *hPYGO2* promoter were not significant. Furthermore, these results support earlier findings (Figure 3.10 C) that the binding of ER α or SP1 to the *hPYGO2* promoter is not dependent on, or significantly affected by the binding of SP1 or ER α , respectively.

To assess what effects total SP1 protein levels have on the binding of SP1 and ER α to the *hPYGO2* promoter, I knocked down SP1 in MCF7 cells using RNAi. Samples were subsequently processed for ChIP analysis. Crosslinked DNA was immunoprecipitated with IgG, SP1 or ER α antibodies and then subjected to Q-PCR to amplify *hPYGO2* promoter sequence.

Treatment of MCF7 cells (grown in serum supplemented media) with a previously published SP1 targeting siRNA (siSP1) (Abdelrahim et al., 2002) caused a 72.8% ($p < 0.01$) reduction in SP1 occupancy of the *hPYGO2* promoter and a 72.7% ($p < 0.01$) reduction in SP1 binding to the *CTSD* promoter, relative to the non-target control siRNA (siNTC) (Figure 3.12 A). Both of these reductions in SP1 were significant and did not alter ER α binding to either the *hPYGO2* or *CTSD* promoters. Consistent results demonstrating the ER α occupancy on the *hPYGO2* promoter in the absence of SP1 were also obtained in MC2 and BT-474 cells (Figure 3.12 B and C).

Figure 3.12 Determination of the SP1 requirement for ER α binding to the *hPYGO2* promoter in ER α + BrCa cell lines.

MCF7 (A), MC2 (B) and BT-474 (C) cells were treated with SP1 directed (siSP1) or non-target control siRNA (siNTC) and ChIP assays were performed to measure ER α and SP1 occupancy at the *hPYGO2* and *CTSD* promoters after E₂ treatment. In all cases promoter occupancy was analyzed by Q-PCR and normalized to IgG in the siNTC sample and adjusted accordingly in the remainder of the samples. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=).



These findings suggested that SP1 binding to the *hPYGO2* promoter depends primarily on total protein levels present within cells. Moreover, these results suggested that ER α binding to the *hPYGO2* promoter is not dependent on, or significantly affected by, the presence of SP1, as observed earlier (Figure 3.10 C)

3.3.9 Estrogen receptor alpha and SP1 transcription factor bind to the hPYGO2 promoter through their respective DNA binding domains

The independent binding of ER α and SP1 to the *hPYGO2* promoter and the requirement of both the ERE half-site and the GC-box suggested that promoter binding by these factors is mediated by their respective DBDs.

To evaluate the requirement of the DBDs for the promoter occupancy of these factors, I performed ChIP assays using site-directed mutant ER α and SP1 expression vectors. The nucleotide (and subsequently amino acid) changes to ER α and SP1 cDNA sequences were specific and critical to the structural composition and DNA binding capacity of the DBDs (Saegusa et al., 1997; DeNardo et al., 2007).

MCF7 cells deprived of hormones were transfected with different combinations of wildtype and mutant expression vectors. These combinations included: wildtype (ER α +SP1), ER α DBD mutant (DBM) (ER α -DBM+SP1), SP1 DBM (ER α +SP1-DBM) and both ER α and SP1 DBMs (ER α -DBM+SP1-DBM). The DNA binding capacity of these expression vectors was measured on pGL3-basic (empty construct negative control) and on pGL3-531 (constructs containing the wildtype ERE half-site and GC-box). After

transfection, cells were treated with E₂ and processed for ChIP analysis. ChIP assays were performed using IgG, ER α and SP1 antibodies and isolated DNA was quantified by Q-PCR. The IgG, ER α and SP1 values for the pcER α + pcSP1 sample in pGL3-basic were all set to 1 for comparative purposes.

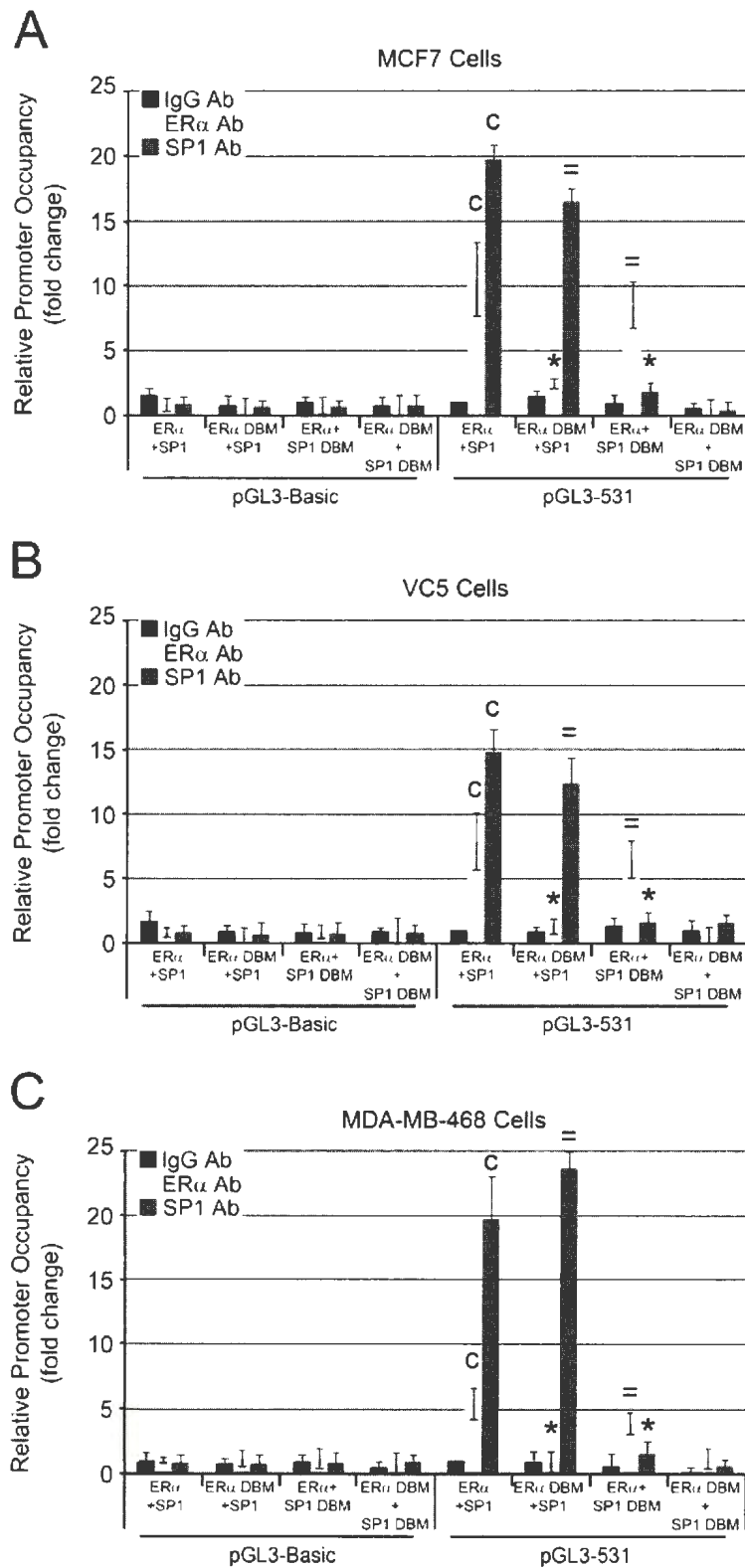
No significant binding relative to the IgG control was observed for any of the ER α and SP1 expression vectors on the pGL3-basic reporter construct (Figure 3.13 A). Overexpression of ER α and SP1 caused an 10.6 fold ($p=0.015$) increase in ER α binding and an 19.7 fold ($p<0.01$) increase in SP1 binding to the pGL3-531 reporter construct relative to the IgG control and was comparable to the binding strength observed earlier (Figure 3.10 C and 3.12 A). The overexpression of ER α DBM caused a significant reduction (77.3%, $p<0.01$) in ER α binding, but didn't alter SP1 binding levels, relative to the IgG control. Overexpression of the SP1 DBM significantly reduced (91%, $p<0.01$) SP1 promoter occupancy while not influencing ER α binding. The overexpression of both ER α and SP1 DBMs completely abolished binding and was not significantly different from the IgG control.

Consistent results demonstrating the requirement of the DBDs of both ER α and SP1 for occupancy of the *hPYGO2* promoter were also obtained in ER α - cell lines, VC5 and MDA-MB-468 (Figure 3.13 B and C).

These findings are consistent with earlier results demonstrating the independent promoter binding of ER α and SP1 to the *hPYGO2* promoter. Additionally, these results

Figure 3.13 Requirement of the ER α and SP1 DNA binding domains for *hPYGO2* promoter occupancy in ER α + and ER α - BrCa cell lines.

MCF7 (A), VC5 (B) and MDA-MB-468 (C) cells were transfected with wildtype or DBD mutated ER α and SP1 expression vectors and their binding to the pGL3-531 *hPYGO2* reporter vector or an empty vector control (pGL3-basic) was measured by ChIP after 1 h of E₂ treatment. In all cases promoter occupancy was analyzed by Q-PCR and normalized to IgG binding to the pGL3-531 reporter vector after transfection with wildtype ER α and SP1 and adjusted accordingly in the remainder of the samples. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=).



suggested that the mechanism by which ER α and SP1 bind to the *hPYGO2* promoter is through their respective DBDs.

3.3.10 SP1 transcription factor requirement for hPygopus2 expression and cell proliferation in Estrogen receptor alpha negative breast cancer

The independent binding of SP1 to the *hPYGO2* promoter coupled with its requirement via its binding element (GC-box) for maximal *hPYGO2* promoter activity suggested that SP1 may play an important role in hPygopus2 expression in BrCa cells. To gain some information about the requirement of SP1 for hPygopus2 expression, I knocked it down using RNAi in MDA-MB-231 (ER α -) cells in addition to MCF7 (ER α +) cells. The knockdown in ER α - BrCa cells is particularly important as SP1, like hPygopus2, is overexpressed in these cells and may be utilized as a therapeutic target.

Treatment with siSP1 effectively reduced SP1 protein levels in both MCF7 and VC5 cells compared to the non-target control siRNA (siNTC) (Figure 3.14 A and B). The reduction of SP1 was associated with a decrease in hPYGO2 protein levels while not affecting levels of ER α protein in MCF7 cells (Figure 3.14 A). These results suggested that SP1 does indeed play an important role in hPYGO2 expression in both ER α +

and ER α - BrCa cells.

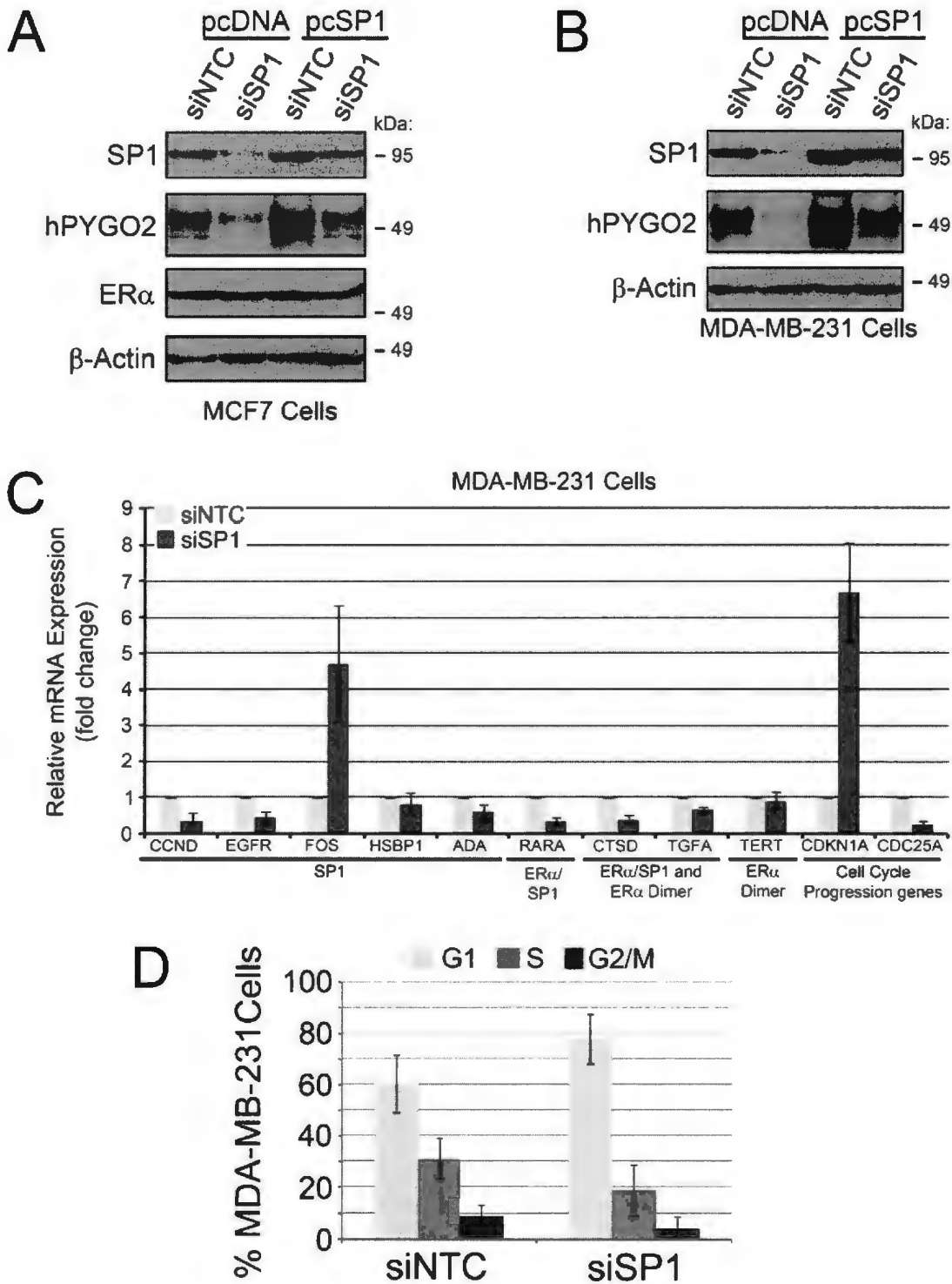
The prevalent requirement of SP1 in the absence of ER α for the expression of hPYGO2, suggested that SP1 may similarly exhibit continued regulation of other E₂ responsive “ER α -SP1” target genes in ER α - BrCa cells.

To assess the requirement of the SP1 transcription factor in the stimulation of “ER α -SP1” target genes, I knocked down SP1 in MDA-MB-231 cells using RNAi. Genes were selected based on the mechanism by which ER α and/or SP1 bind to their promoters and categorized into four groups: SP1 (ER α indirect binding via SP1), ER α -SP1 (direct binding of both ER α and SP1), multiple (combination of both direct and indirect binding) and ER α (ER α dimer binding). I also included two cell cycle progression genes to understand what effect SP1 protein knock down was having on cell proliferation. mRNA was extracted from siRNA treated cells and gene expression was analyzed by Q-PCR.

Treatment of MDA-MB-231 cells with siSP1 caused a reduction in three of the five SP1 group genes (Figure 3.14 C). Cyclin D, Epidermal growth factor receptor and Adenosine deaminase all displayed significant reductions in mRNA expression after siSP1 treatment relative to the siNTC control. While expression slightly decreased for Heat shock factor binding protein 1 due to siSP1 treatment, it was not significantly different from the control. Unexpectedly *FOS* mRNA exhibited increased expression. All the genes in the ER α -SP1 group (*RARA*) and the multiple group (*CTSD* and *TGFA*) were significantly reduced by treatment with siSP1. As expected, Telomerase reverse transcriptase, the gene in the ER α dimer group was not affected by siSP1 treatment. SP1 knockdown also caused a significant increase in the mRNA expression of the *p21* (*CDKN1A*) and a significant decrease in the mRNA levels of the Cell division cycle 25 homologue A.

Figure 3.14 Requirement of SP1 for the expression of ER α target genes and cell cycle progression in ER α - BrCa cells.

Expression levels of hPYGO2, SP1 and ER α protein as measured by immunoblot in MCF7 (A) and VC5 (B) cell lines after treatment with an SP1 targeting siRNA or a non-target control siRNA. β -Actin was used as a loading control and molecular weight is expressed in kDa. (B) Expression levels of SP1 and/or ER α target gene (described in text) mRNA levels as analyzed by Q-PCR in VC5 after SP1 knockdown. mRNA levels were normalized to *β -Actin* and set to one in siNTC treated cells treated samples and adjusted accordingly for other time points. (D) VC5 cells were treated with SP1 targeting siRNAs or the non-target control siRNA and processed for cell cycle analysis.



These results suggested that SP1 modulates the activity of several GC-box containing genes, like hPYGO2, in ER α - BrCa cells despite the absence of ER α . Additionally, the modulation of cell cycle genes like *CCND*, *p21* and cell division cycle 25 homologue A by siSP1 suggested that SP1 may play a role in proliferation of ER α - cells like it has been shown to do in MCF7 ER α + cells (Lu and Archer, 2010).

The finding that SP1 was required for the expression of several cell cycle genes and hPygopus2, a protein that has repeatedly been shown to be required for cancer cell proliferation (Popadiuk et al., 2006; Andrews et al., 2007, Wang et al., 2010, Chen et al., 2011; Watanabe et al., 2013), suggested that SP1 knockdown would result in cell cycle arrest of MDA-MB-231 cells. MDA-MB-231 cells were therefore transfected with siSP1 and the effects on cell proliferation, relative to siNTC, were analyzed by flow cytometry.

When transfected with siNTC, the proportion of cells that accumulated in G1 was 61.8%, but appreciably increased to 75.8% after siSP1 treatment (Figure 3.14 D). The increase was not significant but consistent with the findings of others in these cells (Abdelrahim et al., 2002). The increase of cells in G1 was accompanied by a significant decrease in the percentage of cells in S-phase ($p=0.037$). These results suggested that SP1 protein expression is required for the proliferation of ER α - BrCa cells.

3.4 Discussion

The E₂-mediated enhancement of hPygopus2 through direct binding of the ER α -SP1 complex to the *hPYGO2* promoter highlights the important role of hPYGO2 in BrCa pathogenesis. This builds on our previous findings demonstrating ELF1-mediated activation of *hPYGO2*. The activation of *hPYGO2* by multiple factors (ELF1 and E₂ via ER α -SP1) is not that surprising considering its well established overexpression and requirement for proliferation of BrCa cells.

While overexpression of both ER α and SP1 and subsequent treatment with E₂ led to the highest *hPYGO2* promoter activation and binding and hPygopus2 levels, I found that each of these factors could bind independently of each other. This is a novel result as ER α and SP1 have been shown to bind as a complex at the promoters of other target genes, notably *CTSD*, *TGFA* and *RARA*. While unexpected, this individual binding is corroborated by several observations obtained in this study. Overexpression of either ER α (with E₂ treatment) or SP1 increased the *hPYGO2* promoter occupancy of these factors and enhanced hPygopus2 expression. Inhibition of ER α or knockdown of SP1 did not significantly affect the binding of SP1 or ER α , respectively. Furthermore, mutating the ERE half-site or the GC-box did not affect promoter occupancy of SP1 or ER α , respectively. Additionally, mutating the DBDs of either ER α or SP1 did not affect *hPYGO2* promoter binding of SP1 or ER α , respectively. Lastly, promoter occupancy of SP1 was persistent in hormone deprived conditions (before E₂ treatment) in the absence

of ER α . The observed low levels of hPygopus2 expression in this case are most likely due to presence of inhibitory cofactors and/or lack of stimulatory cofactors.

This type of independent binding suggests that while ER α and SP1 can both recruit the required transcription components to enhance hPygopus2 expression, the presence of both is required for maximal synergistic activation via either optimal recruitment of cofactors or recruitment of all cofactors. This supports the lower levels of hPygopus2 observed in ER α - cells, where SP1 still plays a role in *hPYGO2* induction, but not to the levels observed in the presence of both SP1 and ER α (ER α + cells).

While the specific function of hPYGO2 in BrCa was not examined, the fundamental role of Wnt in BrCa initiation and progression is undeniable, thus a role for hPYGO2 in Wnt signalling is likely. This is supported by a recent study, which showed the requirement of hPYGO2 in augmenting Wnt signalling which contributed to BrCa stem-like cell expansion (Chen et al., 2010). Furthermore, we have shown the interaction between hPYGO2 and Treacher Collins Franceschetti syndrome 1 in MCF7 BrCa cells and demonstrated the importance of this interaction in ribosomal DNA transcription (Andrews et al., Accepted), which may be an additional function of hPYGO2 in BrCa cells.

The overexpression and requirement of hPYGO2 in ER α - (in addition to ER α +) BrCa cells was previously highlighted. While it may have been thought that induction of *hPYGO2* in these cells was due to ELF1, I now provide evidence that SP1 (in addition to ELF1) plays an important role. I demonstrated that overexpression of SP1 increased hPygopus2 levels while knockdown of SP1 reduced hPygopus2 levels in ER α - BrCa

cells. While these findings are novel, they are not surprising considering that SP1 doesn't require ER α to bind to the *hPYGO2* promoter and induce *hPYGO2* in ER α + cells.

My second finding that SP1 is required for the expression of *hPYGO2* and several other cell cycle progression genes (including "ER α -SP1" target genes) in ER α - BrCa is particularly important because of the lack of endocrine disruptor therapies for this cancer.

This is fortunate as several effective drugs that target SP1 exist (Betulinic acid, Mithramycin A and Tolfenic acid) (Leask, 2012). Thus, SP1 may be an important predictive biomarker for selecting the type of chemotherapy. This would be particularly important for ER α - BrCa (due to the lack of effective therapies), but could also be used for ER α + BrCa, either in combination with ER α antagonists and aromatase inhibitors or, alone in cases where traditional therapies don't work because of intrinsic or acquired resistance.

In both cases expression of *hPygopus2* could serve to confirm that SP1 is correctly inducing its target genes and not dissociated from this process. Thus, *hPYGO2* might serve as a predictive biomarker for response to SP1 inhibitors in ER α - BrCa. Additionally, if SP1 expression is reduced by chemotherapy, *hPygopus2* expression would also decrease and thereby should further prevent cancer cell proliferation.

The next step in this study would be to examine if *hPYGO2* and SP1 protein levels correlate in ER α + and ER α - breast tumours and to determine if SP1 inhibitors reduce *hPYGO2* protein levels.

**Chapter 4: Human Papillomavirus E7-mediated attenuation of
Retinoblastoma induces hPygopus2 expression through the E74-like
factor 1 in cervical cancer**

4.1 Introduction: Cervical Cancer

4.1.1 Foreword

In the previous chapter, I examined the mechanism of expression of hPygopus2 in breast cancer, and provided evidence to suggest that its requirement for growth of breast cancer cells was linked to the presence of endogenous hormonal and signalling elements that influenced, but did not necessarily cause the malignant phenotype. In this chapter, I present evidence to suggest that hPygopus2 expression is also induced at the earliest stages of oncogenesis, using the example of cervical cancer. My data suggested that hPygopus2 is an important factor in the progression of this disease, and its expression is an early response to the pathogenic agent responsible for cervical cancer, the human papillomavirus (HPV).

4.1.2 The cervix

The cervix is the lower portion of the uterus. The upper region (closest to the uterine cavity) is lined by a single cell thick layer of columnar (glandular) mucus secreting cells and is referred to as the endocervix (Singer and Jordan, 2006) (Figure 4.1). The lower region (closest to the vaginal canal) is covered by a thick multilayered stratified squamous epithelium and is referred to as the ectocervix. The development and maintenance of this epithelium occurs by proliferation of the basal cells (the only proliferation capable cells within the epithelium). As these cells divide, they push cells into the above layers where they undergo a gradual process of differentiation. This

includes flattening out and filling their cytoplasm with keratin granules, while reducing the size/number of organelles and size of nucleus.

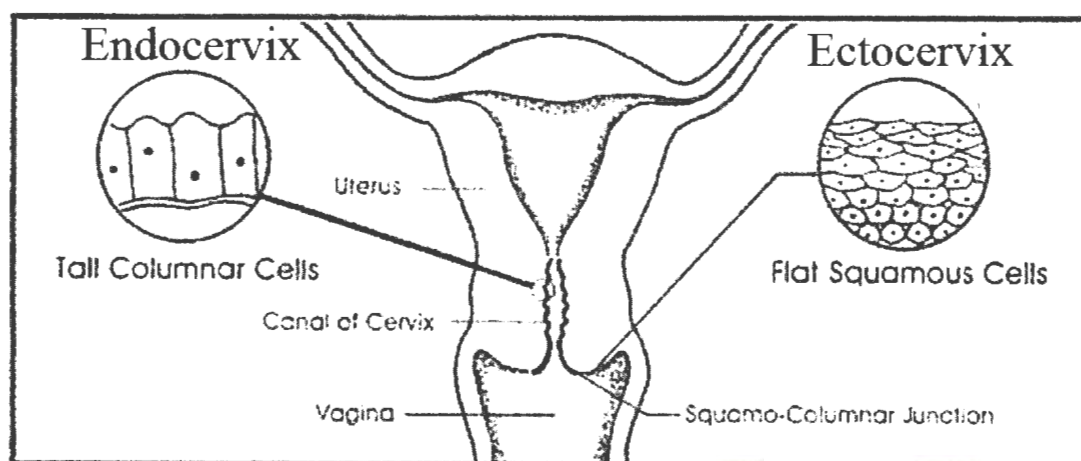
The boundary between the squamous and columnar epithelia is called the squamocolumnar junction. Within this area occur all the dynamic, physiological and pathological processes that are found in the human cervix (Singer and Jordan, 2006). Thus, this region is referred to as the transformation zone and 90% of precancerous and cancerous lesions occur here.

4.1.3 Cervical intraepithelial neoplasia

Progression from the normal cervix to malignant cervical cancer (CxCa) is a multistep process that begins with HPV infection (Moscicki, 2007). Infection stimulates proliferation of cervical cells and results in the formation of cervical lesions which are referred to as cervical intraepithelial neoplasia (CIN) or dysplasia. CIN has been divided into three stages depending on what proportion of the epithelium contains abnormal cells. Low grade lesions (CIN I) will most likely spontaneously regress whereas high grade lesions (CIN II, CIN III and carcinoma *in situ*), are true cervical cancer precursors because they have a high probability of becoming malignant. As mentioned, both types of CIN are caused by HPV infection.

HPV DNA was first isolated from CxCa biopsies almost forty years ago, at which time it was suggested that HPVs could be a potential cause of CxCa (zur Hausen, 1977). Strong clinical evidence, such as the detection of HPV DNA in 99.7% of cervical carcinomas worldwide and experimental evidence, the observation that expression of

Figure 4.1 Schematic diagram showing endo- and ectocervical portions of the cervix and the type of epithelium by which they are covered.



high risk HPVs or the E6 and E7 viral oncogenes could immortalize human keratinocytes (Hawley-Nelson et al., 1989), has demonstrated that the high-risk HPVs are the only required agents of CxCa.

4.1.4 Human papillomavirus classification

According to the International Agency for Research on Cancer, the HPV family of DNA viruses is comprised of over 100 types (Clifford et al., 2005). Thirty-five of these are specific for genital and oropharyngeal epithelia and have varying potentials for malignant transformation. Of these, twelve are low-risk types (including 6, 11), which do not integrate into the host genome and are only associated with lower-grade squamous intraepithelial lesions (LSILs) and benign warts. Fifteen HPV types are considered high-risk types (including 16, 18, 31) and are strongly associated with high grade SILs (HSILs) and invasive carcinoma (Munoz et al., 2003). To understand how these viruses cause disease, we first need to examine their structure and gene composition.

4.1.5 HPV genome structure

The eight kilobase pair double stranded DNA circular HPV genome is contained within a spherical protein coat (Baker and Calef, 1996). The viral genome comprises a region for the six early genes E1–E7, two late genes L1 and L2, and a long non-coding control region.

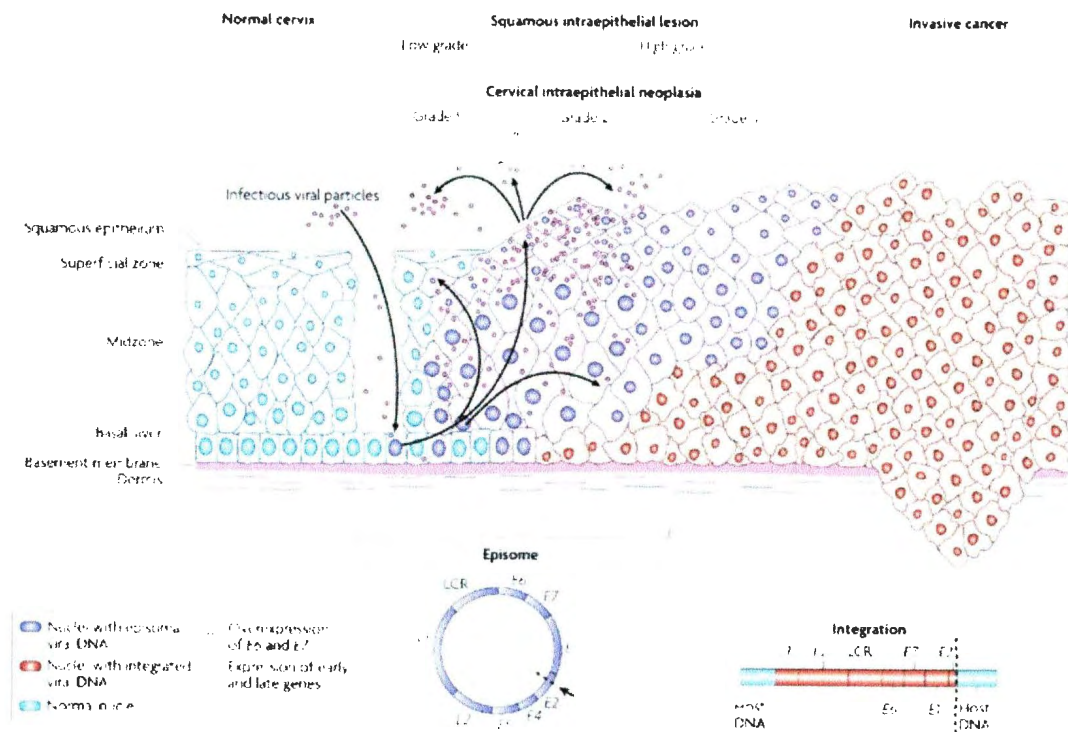
The early genes E1 and E2 enable viral transcription and replication, and E5 and the two well established viral oncogenes (E6 and E7) are responsible for the transforming characteristics. The late genes L1 and L2 code for the viral capsid proteins and the late protein E4 plays a role in release of the newly formed viruses from sloughed off epithelial cells. The viral life cycle begins when the virus infects a proliferation capable cervical cell.

4.1.6 HPV mechanism of infection and normal life cycle

Cell infection and viral replication is characterized very well in the squamous epithelium; while the mechanism of infection is currently unknown for columnar epithelia but is believed to be similar.

For persistent infection to occur, virus particles must penetrate deep within the epithelium through abrasions or other microtrauma and infect basal cells (Moscicki, 2007) (Figure 4.2). Initially, however, the viruses make contact with heparan sulfate proteoglycan receptors on the basement membrane. Binding to these molecules causes a structural shift in the viruses' protein coat and allows the viruses to be taken up by cells. It is important to note that if the viral contact with these receptors is prevented, then the virus will not be able to infect cells. The specific cells that HPV infects are thought to be stem cells because they are the only proliferation competent cells within the epithelium. Furthermore, the highly dynamic nature of the transformation zone makes it prone to microabrasions and thus the stem cells within this area are more susceptible to HPV infections.

Figure 4.2 Schematic diagram highlighting the mechanism of infection by the human papillomavirus in the cervical squamous epithelium (adapted from Woodman et al., 2007).



Once the viral genome, which is maintained as an extrachromosomal element (episome), enters the nucleus of basal cells it begins to express the early viral genes (Moscicki, 2007). The virus utilizes host machinery for replication, with the exception of the E1 viral helicase and the E2 transcription factor which regulates expression of the remainder of viral genes (You et al., 2004). As the virus replicates, it must counteract signals for terminal differentiation of the squamous epithelium and force the keratinocytes into S phase. This is achieved by viral oncoproteins E6 and E7 which have numerous targets (and effects) that contribute to the transforming characteristics, most notably the Tumor protein 53 (p53) and RB tumour suppressors.

E7 binding to the active (hypophosphorylated) form of RB triggers its degradation, which leads to E2F-RB complex dissociation (Masciullo and Giordano, 2007). Activated E2F initiates the transcription of genes required for DNA replication and thus inappropriately forces the cell through the restriction point and past the G1/S transition into S phase. The continuation of this oncogene driven cell division would normally be prevented by p53. However, E6 in a complex with the E6-associated protein binds to and inactivates p53 by targeting it for degradation through the ubiquitin proteasome system. This alleviates the p53 cell cycle arrest induced restrictions on cellular DNA synthesis and augments viral replication (Scheffner et al., 1990).

The action of these oncogenes (by inducing cell division and DNA replication) and the action of E1 and E2 (which replicate the virus during every S phase) permit the

infected basal keratinocytes to over-replicate the viral genome as they leave the basement membrane and progress through their, now delayed, differentiation (Moscicki, 2007).

After sufficient viral replication and terminal differentiation (due to the upward migration through the epithelium) of the keratinocytes, the capsid protein L2 and L1 are expressed (Moscicki, 2007). They recruit the viral genome and assemble to form infectious virions. As the infected squames slough off the epithelial surface the E4 protein causes them to disintegrate and release the virion particles.

4.1.7 Risk factors for progression

Although more than 99% of all cervical cancers are associated with HPV infection, only a fraction of HPV-infected women develop CxCa (Walboomers et al., 1999). This suggests that HPV infection alone is insufficient for the malignant conversion, and that other genetic changes and/or exogenous factors are required for the development of CxCa. One of the required genetic changes for progression is the integration of the viral genome into the host genome.

During the normal viral life cycle, E6 and E7 proteins are maintained at low levels by the transcriptional regulation of E2 (Masciullo and Giordano, 2007). Viral integration disrupts the viral E2 gene (region most susceptible to DNA breaks), thereby negating E2-mediated repression of E6 and E7 and triggering their overexpression. Derepression of E6 and E7 not only suppresses cell-cycle checkpoints, but also exacerbates genomic instability through aberrant centrosomal duplication and leads to the activation of telomerase. The cause of viral genome integration is unknown, but it is

believed that exogenous cell stress inducing factors, such as tobacco, play a role not only in this event, but in other events required for CxCa development (Fonseca-Moutinho, 2011).

4.1.8 Cervical cancer and treatments

Squamous cell carcinoma (SCC) of the cervix accounts for 80-85% of all CxCa and arises from the squamous cells near the transformation zone. The less common (15-20%) cervical adenocarcinomas are often situated deep within the cervical canal. HPV 16 and 18 are associated with 70% of all cervical carcinomas worldwide while the remaining 30% are caused by thirteen other high-risk HPV types.

While the incidence of squamous cell carcinomas has been greatly reduced (by 70%) due to screening initiatives, the incidence of adenocarcinoma has doubled over the past thirty years.

Once cervical cancer is diagnosed, depending on its status, it can be treated with surgery, radiation and chemotherapy. Cisplatin chemotherapy is used with radiation therapy as the first line treatment in bulky and advanced cervical cancer (discussed in the next chapter).

4.1.9 Chapter summary

In Chapter II, I demonstrated that hPygopus2 mRNA and protein exhibit cell cycle dependent expression in a broad array of normal and cancerous cell lines. This

specific pattern of expression and the potential utilization of hPYGO2 as a prognostic proliferation marker provided support for the further examination of hPYGO2 as a cancer biomarker. Specifically, as the cell cycle dependent expression of hPygopus2 correlated with its potential regulation by ELF1 and RB, this was the mechanism of regulation that I chose to pursue.

Not only is the cause and progression of CxCa very well characterized, and thus the exact mechanism by which RB is targeted explicitly known, but ELF1 has also been previously shown to be up regulated in this cancer.

Paradigmatic of pathogenically mediated RB deregulation is the action of HPV E7 protein, a key etiological agent in the initiation of CxCa (Moody and Laimins, 2010). E7 is expressed following infection of proliferation-competent cervical cells with certain high-risk HPV subtypes. Similar to other viral proteins such as papovavirus T antigen and adenovirus E1A (Pahel et al., 1993), E7 is a 98 amino acid nuclear phospho-oncoprotein, which cooperates with the HPV E6 protein to cause immortalization of epithelial cells (Hawley-Nelson et al., 1989). E7 induces degradation of the RB protein, resulting in the activation of several transcriptional regulators such as E2F and ELF1, which promote cell cycle progression (Chellappan et al., 1992).

I was interested in understanding the mechanism of expression and requirement of hPygopus2 in CxCa. HPV genome integration in abnormally proliferating cervical cells raises the probability that they will progress to malignancy because the HPV E2 genomic region is spliced out and the HPV E2 regulatory protein is no longer produced (Schwarz et al., 1985). The loss of E2 causes the constitutive expression of E6 and E7. E6 recruits

the cellular E3 ubiquitin ligase E6-associated protein and forms a trimeric complex with p53 (Huibregtse et al., 1991) leading to proteasomal degradation (Scheffner et al., 1990), and preventing cell cycle arrest and/or apoptosis. The E7 protein, through the LxCxE motif, binds to the pocket region of RB and drives its continuous proteolytic degradation (Munger et al., 1989), permitting ELF1 to initiate expression of target genes. I hypothesized that the increase in hPygopus2 expression required for growth of transformed cervical cells may be the result of ELF1 activation caused by HPV E7-mediated attenuation of RB.

In this chapter, I will provide evidence that hPygopus2 is overexpressed in CxCa and is required for growth of transformed HPV-infected human endo- and ectocervical cells. My data indicated that hPygopus2 overexpression in CxCa is augmented by abrogation of RB function by E7 leading to derepression of ELF1, suggesting a mechanistic link between HPV infection and cellular response of the oncogenic hPYGO2 transcription factor.

4.2 Materials and Methods

4.2.1 Tumor microarray, immunohistochemistry and analysis

Immunohistochemistry was performed on cervical tumour microarrays [Cybridi, Lot No. CC10-11-004 (192-194) and US Biomax, Inc., Cat No. CIN481] using Ventana Benchmark Ultra automated clinical immunostainers optimized for the antibodies indicated as per manufacturer's instruction or as indicated in appendix Table 6.2.

For the Cybridi microarray, three to four representative core samples for each stage (per antibody) were selected for quantification of stained cells. Within each core, at least 100 randomly selected cells throughout the thickness of the epithelium were scored. The overall proportion of stained cells for HPV L1 was assessed. For hPYGO2, PCNA and ELF1, the overall proportion of cells was categorized as nuclear, cytoplasmic or both.

For the US Biomax microarray, three to four representative core samples for each stage (per antibody) were selected for quantification of stained cells. Within each core, at least 100 randomly selected cells throughout the thickness of the epithelium were scored. Staining intensity for each antibody (hPYGO2, p16INK4a and MK167) was classified as negative, weak or strong. Information on the statistical analysis is provided in appendix Figure 6.4.

4.2.2 Cell line maintenance

Normal human endocervical (HEN) (Tsutsumi et al., 1992) and ectocervical (HEC) (Yokoyama et al., 1994) primary cells and their HPV 16 or 18 and cigarette smoke condensate transformed subclones, HEN 16T (Yang et al., 1996) and HEC 18T

(Nakao et al., 1996), were generous gifts from Dr. A. Pater. Primary cell lines were maintained in DMEM supplemented with 2 µl/ml bovine pituitary extract (Gibco) and 0.06 µl/ml epidermal growth factor (Gibco BRL) and cancer cells in DMEM plus 10% heat inactivated FBS. Detailed information on cell lines is provided in appendix Table 6.3 and appendix Figure 6.5.

4.2.3 RNA extraction, cDNA generation and Q-PCR

Performed as described earlier (Sections 2.2.8 and 2.2.9) and primer sequences are listed in appendix Table 6.1.

4.2.4 Protein extraction, SDS-PAGE and immunoblotting

Performed as previously described (Section 2.2.10) and antibody information is provided in appendix Table 6.2.

4.2.5 RNA interference

Four siRNA oligonucleotides were synthesized by Dharmacon, including siPy2-X (which targets the 3' UTR sequence of endogenous *hPYGO2* mRNA), siPy2-Z (which targets the coding region of *hPYGO2*) and siE7 (Chang et al., 2010). We have previously described the ELF1 targeting siRNA, siELF1, (Andrews et al., 2008) and the non-specific negative control, siNTC. Oligonucleotide sequences are listed in appendix Table 6.1.

4.2.6 siRNA transfections and rescue assays

Thirty thousand cells per well were seeded in six well plates and forward transfected as described earlier (Section 3.2.12). In rescue assays, cells were additionally

transfected with pCS2+ or pCS2+hPygo2 and harvested as described earlier (Section 3.2.12).

4.2.7 Flow cytometry

Performed as described earlier (Section 2.2.6).

4.2.8 Chromatin immunoprecipitation and DNA extraction

HEN 16T and HEC 18T cells were seeded in 150-mm dishes at a density of 5×10^6 . After 1 d, cells were transiently transfected with combinations of PSM-RB, ELF1 and DA-ELF1 and collected 3 d later. ChIP assays were performed as described earlier (Section 3.2.10). Antibodies used for immunoprecipitation and primers sequences for amplification are listed in appendix Table 6.1 and appendix Table 6.2, respectively.

4.2.9 Plasmids

pECE-PSM-RB, was provided by Dr. Brenda Gallie (Hamel et al., 1992). The pECE-ΔKPN vector control for pECE-PSM-RB was generated by digesting pECE-PSM-RB with KpnI and re-ligating to remove the transcriptional start site. pCS2+ELF1 (Andrews et al., 2008) contains the human *ELF1* cDNA and the RB binding deficient mutant, DA-ELF1, was generated by site-directed mutagenesis as described earlier (Section 3.2.7) according to sequences in (Wang et al., 1993). pCS2+ was used as the empty vector control for both ELF1 plasmids.

hPYGO2 reporter constructs pGL3-1494 antisense, pGL3-1494, pGL3-48 and pGL3-basic were previously described (Andrews et al., 2008). Site-directed mutagenesis

was used to generate the EBS mutant pGL3-48 mutEBS according to sequences in (Gory et al., 1998). Plasmids were verified by sequencing. Primer sequences are listed in appendix Table 6.1.

4.2.10 Transient transfections and luciferase assays

Cells were seeded 1 d prior to transfection using Lipofectamine and Plus reagent in six well plates (3.0×10^5 cells/well) using 1 μ g of DNA (per well) or in 15 cm plates (7.5×10^6 cells) using 5 μ g of DNA. Three types of transfections were performed: 1) 2 expression plasmids (pECE-PSM-RB, pCS2+DA-ELF1, pCS2+ELF1) and their empty plasmid counterparts (0.5 μ g of each per well); 2) 1 luciferase reporter plasmid (pGL3-1494 antisense, pGL3-1494, pGL3-48, pGL3-48 mutEBS or pGL3-basic) with pRSV- β -gal (0.5 μ g of each per well or 2.5 μ g of each per plate); 3) 2 expression plasmids (0.25 μ g of each per well), 1 luciferase reporter plasmid (0.25 μ g per well) and pRSV-bgal (0.25 μ g per well). Luciferase assays were performed as described earlier (Section 3.2.9).

4.2.11 Image acquisition and densitometry analysis

Performed as described earlier (Section 2.2.11).

4.2.12 Statistical analysis

Performed as described earlier (Section 2.2.12).

4.3 Results

4.3.1 hPygopus2 protein is overexpressed in cervical intraepithelial neoplasia III and squamous cell carcinoma

The relative expression of hPYGO2 in HPV infected cervical tissues was assessed using a microarray of tissue core samples representing various stages of disease progression, stained by immunohistochemistry, with antibodies against HPV L1, hPYGO2, PCNA and ELF1. Overall proportions of stained cells were assessed for each marker, as well as proportions of cells staining nuclei or cytoplasm exclusively or both for hPYGO2, PCNA and ELF1.

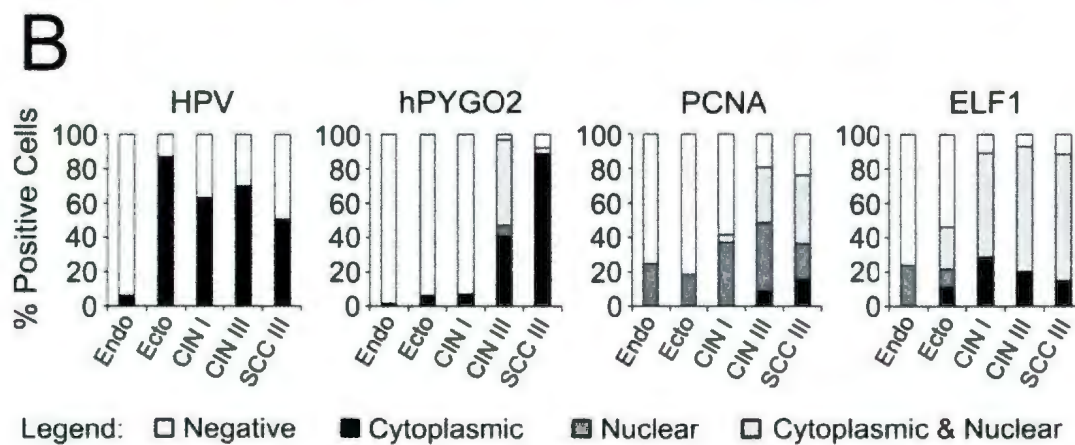
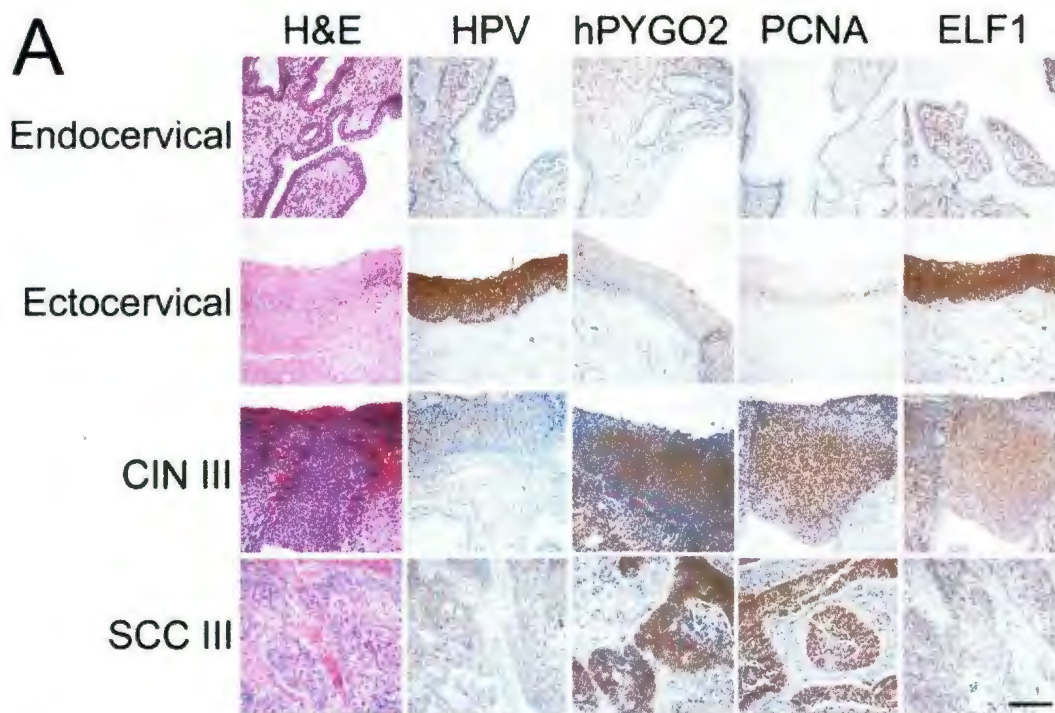
Consistently, HPV was detected in normal ectocervical cells as well as dysplastic and malignant tissue. The staining intensity using this antibody was higher in non-diseased ectocervical but not in endocervical epithelia (Figure 4.3 A and B) (Balan et al., 2010). The proportion of HPV positive cells did not vary significantly.

hPYGO2 protein was detected in both normal and pathologic tissues, with the intensity of staining appreciably higher in CIN III and SCC cores. The proportion of positively stained cells increased significantly from 7% in normal tissues to 93% in severely dysplastic (CIN III) and malignant tissues. hPYGO2 cellular localization was either solely cytoplasmic or both cytoplasmic and nuclear.

Both the expression and staining intensity of the proliferation marker nuclear antigen (PCNA) (Yu and Filipe, 1993), significantly increased with severity of disease, rising from approximately 20% in normal epithelia and 41.5% in mild dysplasia (CIN I)

Figure 4.3 Expression and quantification of HPV, hPYGO2, PCNA and ELF1 proteins in a progressive cervical tumour microarray.

(A) Representative cores of normal ectocervical and endocervical tissues, CIN3 lesions and SCC 3 tumours stained with antibodies against HPV L1, hPYGO2, PCNA and ELF1 are displayed. (B) Three or four representative cores per antibody per condition were selected. One hundred cells were counted in each core and classified as no staining (negative), cytoplasmic staining, nuclear staining or both cytoplasmic and nuclear staining. The percentages were calculated and are displayed.



to 81% and 76% in CIN III and SCC, respectively. In positive cores PCNA protein was primarily localized to the nucleus.

ELF1 protein was detected in all tissues and tumours. While staining intensity clearly decreased with disease severity, the proportion of positively stained cells remained high in all epithelia (above 40%) with the exception of normal endocervical cores (24%). As expected, ELF1 was localized to both the cytoplasm and nucleus (Nicol et al., 2008).

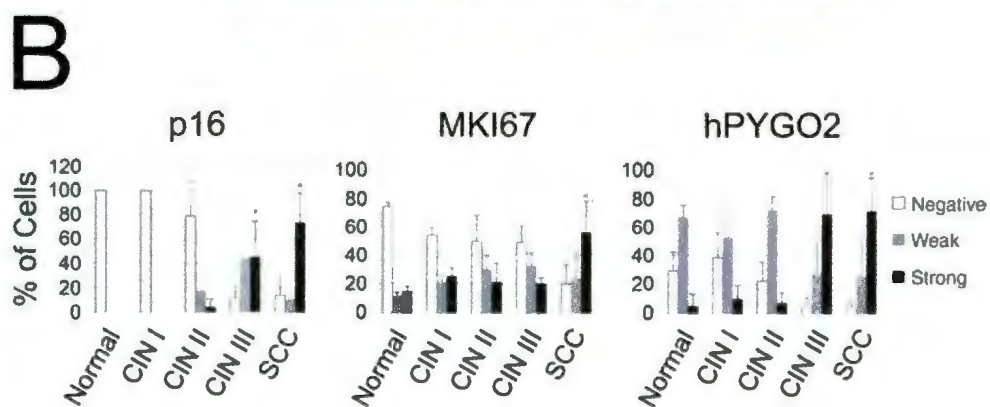
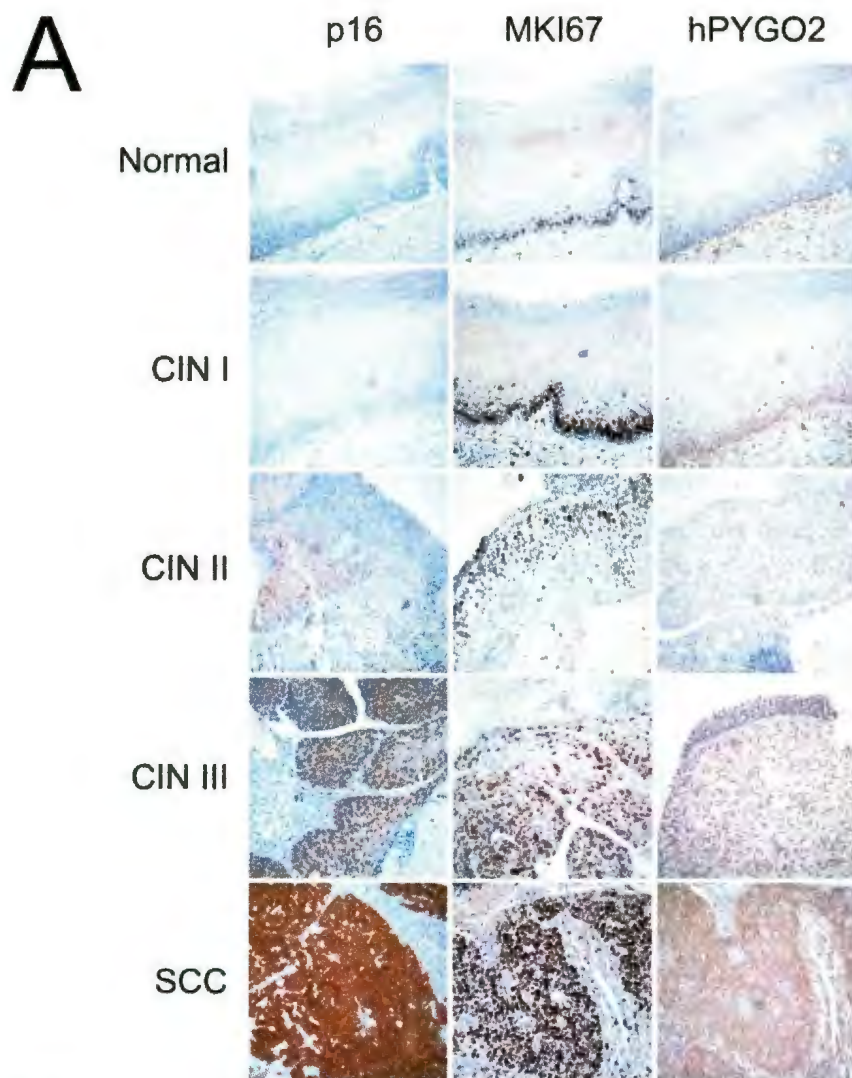
The relatively higher levels of hPYGO2 specifically in CIN III and SCC promoted further examination. I compared the expression of hPYGO2, and two well established diagnostic markers for cervical neoplasia (p16INK4 and antigen identified by monoclonal antibody Ki-67, MKI67) (Gupta et al., 2010), in a CIN tissue array by immunohistochemistry. In this array staining was classified as negative, weak or strong.

Expression of p16 was absent from normal and CIN I cores and slightly increased in CIN II (44% of cells), but staining intensity and proportion was significantly higher ($p<0.02$) in CIN III and SCC cores (89% and 82% of cells, respectively) (Figure 4.4 A and B). The expression of MKI67 increased progressively (14% of cells in normal epithelia, 34% of cells in CIN I, approximately 50% of cells in CIN II and III) with the highest intensity ($p<0.02$), as measured by the percentage of strongly staining cells, in cores representing SCC (78% of cells). Using an ordinal logistic random-effects model (appendix Figure 6.4), it was determined that the intensity of hPYGO2 antibody staining increased significantly with disease progression from CIN II (7% of cells) to CIN III and SCCs (69% and 70% of cells, respectively) ($p<0.05$).

Figure 4.4 Expression and quantification of p16INK4, MKI67 and hPYGO2 proteins in a cervical intraepithelial neoplasia tissue microarray.

(A) Representative cores of normal ectocervical tissues, CIN I, II and III lesions and SCC tumours stained with antibodies against p16INK4, MKI67 and hPYGO2 are displayed.

(B) Three representative cores per antibody per condition were selected. One hundred cells were counted in each core and classified as no staining (negative) or cytoplasmic, nuclear or cytoplasmic and nuclear staining (positive). The percentages were calculated and are displayed. Significant increases in strong staining are denoted with an asterisk (*).



Taken together, these observations suggested that expression of hPYGO2 protein was up regulated in many CIN III lesions and SCC tumours with respect to normal cervical tissue.

4.3.2 hPygopus2 mRNA and protein is overexpressed in cervical cancer cell lines

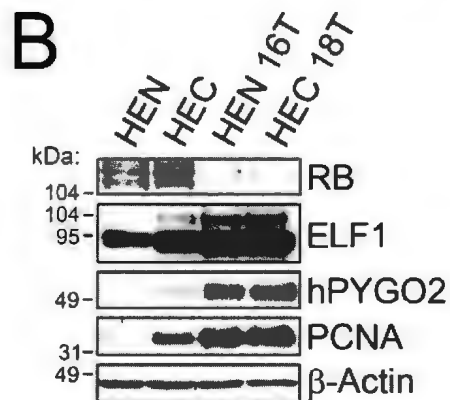
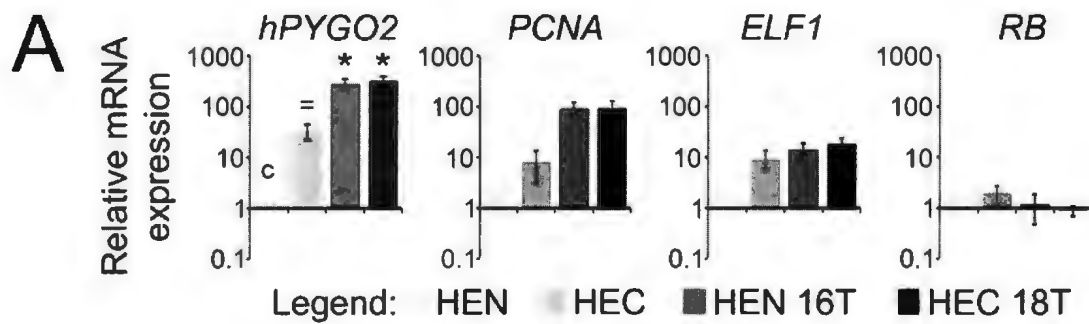
The foregoing observations suggested that increased hPygopus2 expression might be a response to HPV integration and E7 up regulation (occurring in CIN III and beyond). To determine the relationship between hPygopus2 and E7, I performed gene expression analyses in a variety of previously developed normal and transformed cervical cell lines (Tsutsumi et al., 1992; Yokoyama et al., 1994; Yang et al., 1996; Nakao et al., 1996). The control cell lines included human primary HPV-negative endo- (HEN) and ecto- (HEC) cervical cells. The experimental cancer cell lines included HPV-16 and HPV-18 immortalized cells transformed with cigarette smoke condensate, HEN-16T and HEC-18T, respectively. All four human cell lines have been extensively characterized (appendix Table 6.3 and appendix Figure 6.5).

hPYGO2 mRNA expression was slightly higher in HEC cells (12 fold) relative to HEN cells and significantly increased (by at least an additional 9 fold) in HEN 16T and HEC 18T cervical cancer cells (Figure 4.5 A). The expression of *PCNA* and *ELF1* followed the same trend yet increased in HEC and cancerous cell lines. As expected, *RB* tumour suppressor levels were lower in the tumourigenic cell lines. hPYGO2 is detected as two differentially migrating bands on gel electrophoresis, possibly identifying two isoforms, which were observed using a variety of antibodies (data not shown). Both

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Figure 4.5 Expression analysis of hPygopus2 in CxCa cell lines.

(A) Expression of *hPYGO2*, *PCNA*, *ELF1* and *RB* mRNA was analyzed by Q-PCR in normal (HEN and HEC) and malignant cervical cell lines (HEN 16T and HEC 18T). mRNA levels were normalized to β -Actin and set to 2 in HEN cells, adjusted accordingly for the other cell lines and plotted on a logarithmic scale. (B) Expression levels of *hPYGO2*, *PCNA*, *ELF1* and *RB* in normal and malignant cervical cell lines were measured using immunoblots. β -Actin was used as a loading control. Molecular weight is expressed in kDa.



forms also paralleled the corresponding mRNA levels of expression. The protein levels of ELF1 and PCNA closely paralleled mRNA levels (Figure 4.5 B). ELF1 is detected as two bands representing the two post-translationally modified isoforms; the 80 kDa cytoplasmic form and the 98 kDa nuclear promoter-bound form.

These results suggested that hPygopus2 mRNA and protein are higher in HPV transformed cervical cells relative to primary cells and that hPygopus2 expression may be regulated at the transcriptional level.

4.3.3 hPygopus2 and E74-like factor 1 are required for cervical cancer cell proliferation

The higher levels of hPygopus2 observed in HPV infected cells and tissues suggested that it may, as in other cancers (Thompson et al., 2002; Popadiuk et al., 2006; Andrews et al., 2007; Wang et al., 2010; Chen et al., 2011; Brembeck et al., 2011; Watanabe et al., 2013; Moghbeli et al., 2013), have an important function in CxCa. HEN 16T cells were, therefore, transfected with two siRNAs directed against hPygopus2, siPy2-X, targeting its 3' UTR and siPy2-Z targeting the coding region to determine the requirement of hPYGO2. Either siPy2-X or siPy2-Z, significantly reduced both isoforms of hPYGO2 as compared to the non-target control siRNA (siNTC). When transfected with siNTC, the proportion of cells that accumulated in G1 was 33%, but was appreciably higher at 66% ($p<0.01$) after siPy2-X treatment and 55% ($p<0.01$) after siPy2-Z treatment (Figure 4.6 A). The increase of cells in G1 was accompanied by a significant decrease in the percentage of cells in S-phase. HEC 18T cells redistributed in a similar

manner after hPYGO2 siRNA treatment with the largest proportion of cells arresting in G1 (Figure 4.6 B).

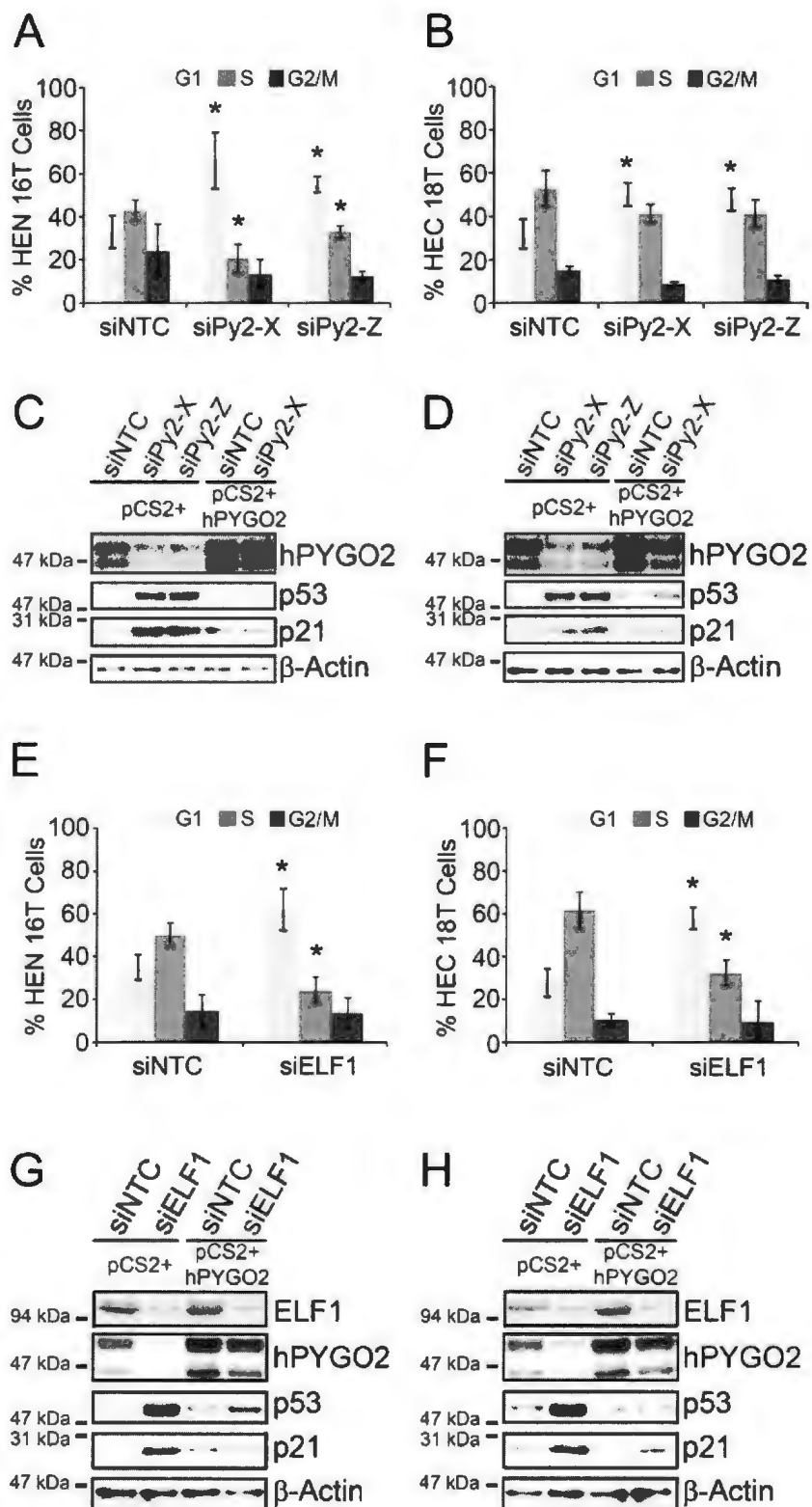
Associated with G1 dependent cell cycle arrest is the activation of the tumour suppressor protein 53 (Waldman et al., 1995). Indeed, higher levels of p53 and its target gene p21 coincided with hPYGO2 knockdown-mediated, G1-phase cell cycle arrest in HEN 16T and HEC 18T cells (Figure 4.6 C and D). Interestingly, the increase in p53 levels was reduced to normal levels by transiently co-transfecting cells with hPYGO2, suggesting that the siRNA was specific for hPygopus2 and that the elevated levels of hPYGO2 protein are a requirement for the proliferation of HPV transformed HEN 16T and HEC 18T cervical cells.

Our previous work demonstrated that the oncogenic viral-ETS family transcription factor ELF1 bound to the *hPYGO2* promoter and could promote its expression in MCF7 BrCa cells (Andrews et al., 2008). I therefore used RNA interference to determine if ELF1 was involved in hPYGO2-dependent cervical cancer cell proliferation. siELF1 increased the proportion of HEN 16T cells in G1 by 27% ($p < 0.01$) relative to siNTC (Figure 4.6 E), which was accompanied by a significant decrease in the proportion of cells in S phase. siELF1 treatment of HEC 18T cells had a similar effect, increasing the proportion of cells in G1 by 34% ($p < 0.01$) (Figure 4.6 F).

In both HEN 16T and HEC 18T cells, the reduction of ELF1 protein levels by siELF1 paralleled a significant decrease in hPYGO2 protein expression (Figure 4.6 G and H). Similar to my results with the hPYGO2 knockdown, there was an increase in p53 and p21 expression upon depletion of ELF1. The siELF1-induced increase in p53 was

Figure 4.6 Requirement of hPYGO2 and ELF1 for the prevention of p53-mediated G1 arrest in CxCa cells.

(A-B) HEN 16T and HEC 18T cells were treated with either non-targeting control (siNTC) or hPYGO2 siRNAs. Flow cytometry was performed to show the percentage of cells in each phase of the cell cycle. (C-D) HEN 16T and HEC 18T cells were treated with either siNTC or hPYGO2 siRNA in combination with either pCS2+ or pCS2+hPYGO2 expression vectors. (E-F) HEN 16T and HEC 18T cells were treated with either siNTC or ELF1 siRNA (siELF1) and subsequently analyzed by flow cytometry. (G-H) HEN 16T and HEC 18T cells were treated with either siNTC or siELF1 in combination with either pCS2+ or pCS2+hPYGO2. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample, are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=).



attenuated back to control levels by overexpression of hPYGO2 (Figure 4.6 G and H), suggesting that ELF1-mediated activation of hPYGO2 expression was required for cell cycle progression in these cells or that overexpression of hPYGO2 could somehow relieve the p53-induced stress response.

4.3.4 Retinoblastoma dependent regulation of hPygopus2 expression via E74-like factor 1 is deactivated by human papillomavirus E7 protein

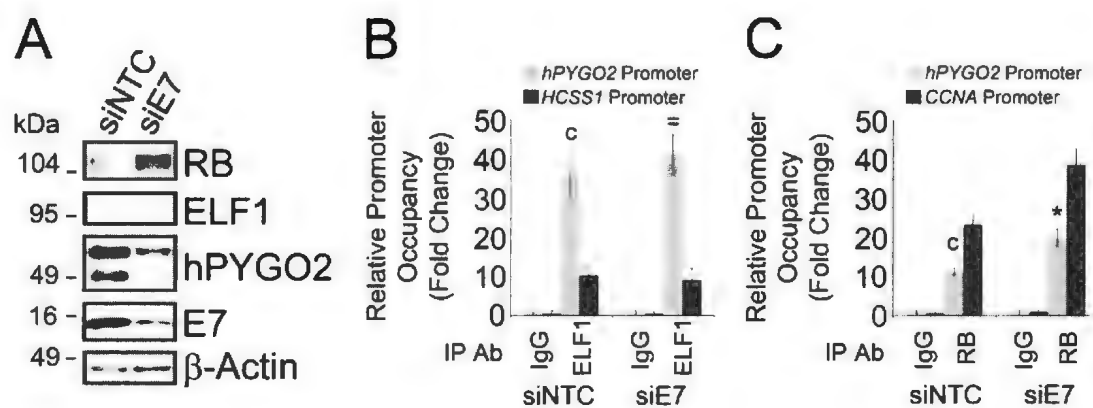
The requirement of ELF1 and hPYGO2 in CxCa suggested that they are activated following HPV integration. A critical effector for HPV pathogenesis is the deregulated expression and activity of its E7 viral oncoprotein. Since E7 induces degradation of RB, I predicted that up regulation of RB by reducing E7, would be expected to decrease ELF1 activity, leading to *hPYGO2* down regulation.

To assess whether E7 was required for increased ELF1 activity and subsequently hPygopus2 expression, I knocked down E7 protein using RNAi. Treatment of HEN 16T cells with an E7-specific siRNA siE7 effectively reduced E7 protein levels compared to the non-target control siRNA (Figure 4.7 A). The reduction of E7 was associated with a decrease in hPYGO2, but an increase in RB protein levels, while not affecting levels of ELF1. It is possible, therefore, that RB attenuated hPYGO2 levels by regulating the activity but not the expression or stability of ELF1.

ELF1 is an ETS-related transcription factor that binds to a number of target genes including the *hPYGO2* promoter whose expression is augmented in cancer (Scott et al., 1994; Zhu et al., 2006; Popadiuk et al., 2006; Jin et al., 2009). *In vitro* protein interaction

Figure 4.7 Requirement of HPV 16 E7-mediated reduction of RB to derepress the *hPYGO2* promoter in HEN 16T cells.

(A) HEN 16T cells were treated with either a non-targeting control (siNTC) or an E7 siRNA (siE7). Immunoblots were performed to confirm the knockdown of E7 protein and levels of RB, ELF1 and hPYGO2 were determined. β -Actin was used as a loading control. (B-C) Sheared, cross-linked chromatin extracts from HEN 16T cells treated with either siNTC or siE7 were subjected to ChIP assays using antibodies against RB (B) and ELF1 (C). A rabbit and mouse IgG mixture was used as a negative control. Promoter regions of *hPYGO2*, *HCCS1* and *CCNA* were amplified by Q-PCR. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=).



assays demonstrated that RB represses ELF1 by binding to its transactivation domain in non-dividing cells, attenuating its ability to activate target gene expression (Wang et al., 1993). Since RB is a major degradation target of HPV-E7, I determined whether knockdown of E7 affected its presence, along with ELF1, at the *hPYGO2* promoter using ChIP assays. We previously examined a 1,568 bp (-1,494 to +74) segment of the *hPYGO2* promoter and demonstrated that ELF1 binds to the -48 to -25 region which contains an EBS (Andrews et al., 2008). After siNTC treatment of HEN 16T cells, the presence of ELF1 and RB was detected at the *hPYGO2* promoter and at the promoters of a known ELF1 target gene, *HCCSI* (Zhu et al., 2006) (Figure 4.7 B) and an RB target gene, *CCNA* (Strobeck et al., 2000) (Figure 4.7 C). Treating cells with siE7 did not affect ELF1 binding to the *hPYGO2* or *HCCSI* promoters. There was, however, a significant increase in the presence of RB at both the *hPYGO2* (56%, $p=0.038$) and the *CCNA* promoters. Thus, siE7 increased the association of RB at the *hPYGO2* promoter while not affecting ELF1. This finding suggested that activation of *hPYGO2* by HPV in cervical cancer cells was due to derepression of ELF1 activity by the repressive action of E7 on RB.

To further analyze the derepression of *hPygopus2* by HPV E7 I utilized a gain of function mutant of RB (PSM-RB) (Hamel et al., 1992), a dominant acting RB-independent mutant of ELF1 (DA-ELF1) (Wang et al., 1993) and wildtype ELF1 and assessed their effect on *hPygopus2* expression. The PSM-RB construct has eight phosphorylation sites substituted by alanines, making it refractory to cyclin-CDK complexes and thus rendering it constitutively repressive (Hamel et al., 1992). Protein

products synthesized using this construct are still able to bind ELF1. The DA-ELF1 construct has the three crucial amino acids in its LxCxE, which are required for binding to RB, substituted with RxRxH, rendering it unable to interact with RB (appendix Figure 6.6). I hypothesized that overexpression of PSM-RB would reduce the positive effect of E7 on hPygopus2 expression, and in contrast, the DA-ELF1 mutant would constitutively activate hPygopus2 expression.

Our previous work indicated that ELF1 augmented *hPYGO2* gene expression by binding to the EBS present within the first 48 base pairs of the *hPYGO2* promoter (Andrews et al., 2008). To assess the effect of the RB and ELF1 constructs on *hPYGO2* gene regulation I overexpressed different combinations of PSM-RB, ELF1 and DA-ELF1 in conjunction with two reporter constructs, both of which included the EBS but differed in the length of *hPYGO2* promoter DNA. These included the minimal *hPYGO2* promoter containing the first 48 base pairs (pGL3-48), a large segment containing 1494 base pairs (pGL3-1494) and a negative control with no promoter sequence (pGL3 Basic) (Andrews et al., 2008).

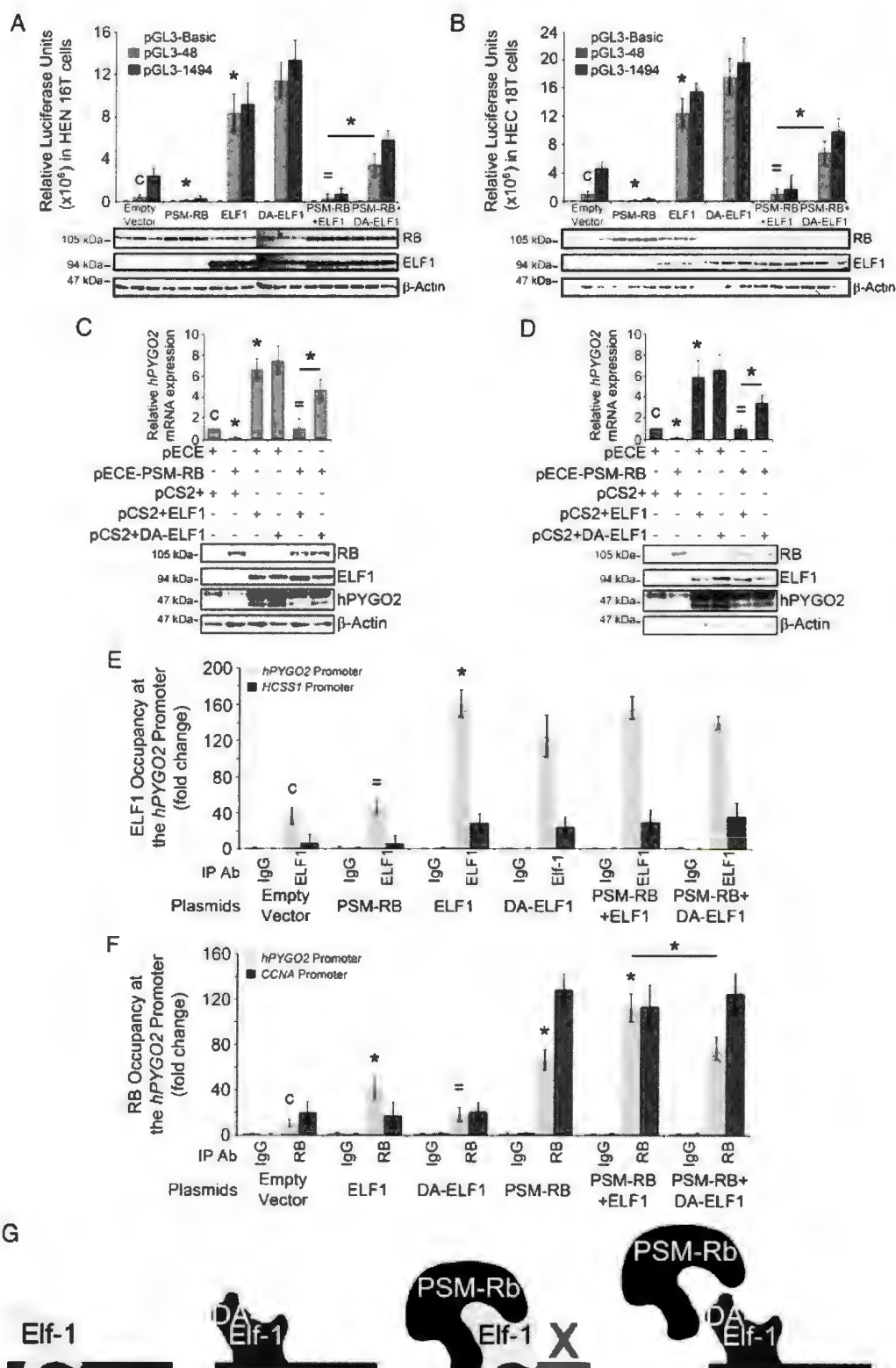
Overexpression of PSM-RB in HEN 16T cells led to 2.9 fold ($p=0.037$) and 7.1 fold ($p=0.038$) decreases in the pGL3-48 and pGL3-1494 samples, respectively, compared to the empty vector control (Figure 4.8 A). In contrast, transfection with either ELF1 or DA-ELF1 significantly increased *hPYGO2* reporter activity compared to the control. ELF1 over-expression caused a 17.6 fold increase ($p=0.017$) in the minimal promoter reporter and a 3.7 fold increase ($p=0.047$) in the large reporter. DA-ELF1 overexpression increased the minimal reporter activity by 24.1 fold ($p<0.01$) and the

large reporter activity by 5.3 fold ($p=0.011$). When PSM-RB and ELF1 were overexpressed in combination, neither the minimal promoter reporter plasmid nor the large promoter reporter plasmid activity was significantly higher than that of the empty vector control. However, when PSM-RB was overexpressed with DA-ELF1 there was a significant increase in both the minimal reporter activity (7.4 fold, $p=0.036$) and the large reporter activity (2.3 fold, $p=0.012$) compared to the control. Overexpression of the RB and ELF1 constructs in HEC 18T cells yielded similar results; RB reduced *hPYGO2* promoter activity of both reporters while ELF1 increased it and DA-ELF1 was able to overcome the repressive effects of PSM-RB (Figure 4.8 B). The similar effects of RB and ELF1 overexpression on both the pGL3-48 and pGL3-1494 reporters suggested that RB and ELF1 modulated *hPYGO2* reporter activity through the EBS in its promoter. Thus, RB negatively, while ELF1 positively modulated *hPYGO2* reporter activity through its EBS.

To examine if RB and ELF1 could modulate endogenous hPygopus2 mRNA and protein levels, I transiently transfected cells with combinations of PSM-RB, ELF1 and DA-ELF1 expression vectors. Overexpression of PSM-RB in HEN 16T cells resulted in a 5 fold ($p<0.01$) reduction in *hPYGO2* mRNA levels and a 53% decrease in hPYGO2 protein levels (Figure 4.8 C). Both ELF1 and DA-ELF1 overexpression increased hPygopus2 mRNA and protein levels by 6 fold. When PSM-RB and ELF1 were co-transfected, PSM-RB inhibited ELF1-induced hPygopus2 expression resulting in no change in hPygopus2 mRNA and protein levels. However, when PSM-RB and DA-

Figure 4.8 hPygopus2 expression and promoter occupancy analysis after RB and ELF1 overexpression in CxCa cells.

(A-B) HEN 16T and HEC 18T cells were transfected with combinations of PSM-RB, ELF1 and DA-ELF1 expression vectors in combination with pGL3-48 and pGL3-1494 luciferase constructs and a β -gal expression vector. Empty pCS2+ and pECE expression vectors and the promoter DNA absent pGL3-Basic luciferase construct were used as negative controls. Luciferase activity was normalized to β -gal activity. Overexpression of RB and ELF1 was confirmed by immunoblots. (C-D) HEN 16T and HEC 18T cells were transfected with combinations of PSM-RB, ELF1 and DA-ELF1 expression vectors and their effect on endogenous hPygopus2 mRNA and protein levels were assessed by Q-PCR and immunoblot, respectively. Overexpression of RB and ELF1 was confirmed by immunoblot. (E-F) HEN 16T cells were transfected with combinations of PSM-RB, ELF1 and DA-ELF1 expression vectors and sheared, cross-linked chromatin extracts were subjected to ChIP assays using antibodies against RB (E) and ELF1 (F). A rabbit and mouse IgG mixture was used as a negative control. The promoter regions of *hPYGO2*, *HCCS1* and *CCNA* were amplified by Q-PCR. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk (*), while non-significant changes ($p > 0.05$) are indicated by equality sign (=). (G) A model explaining how the binding of ELF1, DA-ELF1 and PSM-RB to the *hPYGO2* promoter affects *hPYGO2* gene activation.



ELF1 were co-transfected, DA-ELF1 overcame the repressive effects of PSM-RB resulting in 4.7 fold and 4.2 fold increases in *hPygopus2* mRNA and protein levels, respectively. Parallel results demonstrating RB inhibition and ELF1 induction of *hPYGO2* were obtained in HEC 18T cells (Figure 4.8 D).

The foregoing findings suggested that RB and ELF1 cooperatively modulated *hPYGO2* expression. CHIP assays were performed to determine the presence of RB and ELF1 at the *hPYGO2* promoter. Various combinations of PSM-RB, ELF1 and DA-ELF1 were overexpressed and the presence of RB and ELF1 on chromatin located at the *hPYGO2* promoter was measured. Interestingly, while overexpression of PSM-RB significantly reduced ELF1 dependent *hPYGO2* promoter activity, it did not alter the level of ELF1 at the *hPYGO2* promoter compared to the empty vector control (Figure 4.8 E). In the remaining cases, in which either ELF1 or DA-ELF1 was overexpressed there was a consistent increase (3.3 to 4.3 fold) in ELF1 at the promoter. This increase in ELF1 was not significantly affected by the presence of PSM-RB. Similarly, ELF1 at the *HCCS1* gene promoter only increased when it was overexpressed, which was expectedly independent of PSM-RB overexpression. These results demonstrated that the interaction between ELF1 and RB is not required for ELF1 to bind to the *hPYGO2* promoter.

Given that RB did not influence the association of ELF1 at the *hPYGO2* promoter, I hypothesized that the reciprocal may be true, that its own presence at the *hPYGO2* promoter is dependent on ELF1. While overexpression of PSM-RB resulted in a 6.5 fold increase ($p=0.017$) in RB (both endogenous and exogenous) at the promoter relative to the control (Figure 4.8 F), the highest levels were achieved when PSM-RB was

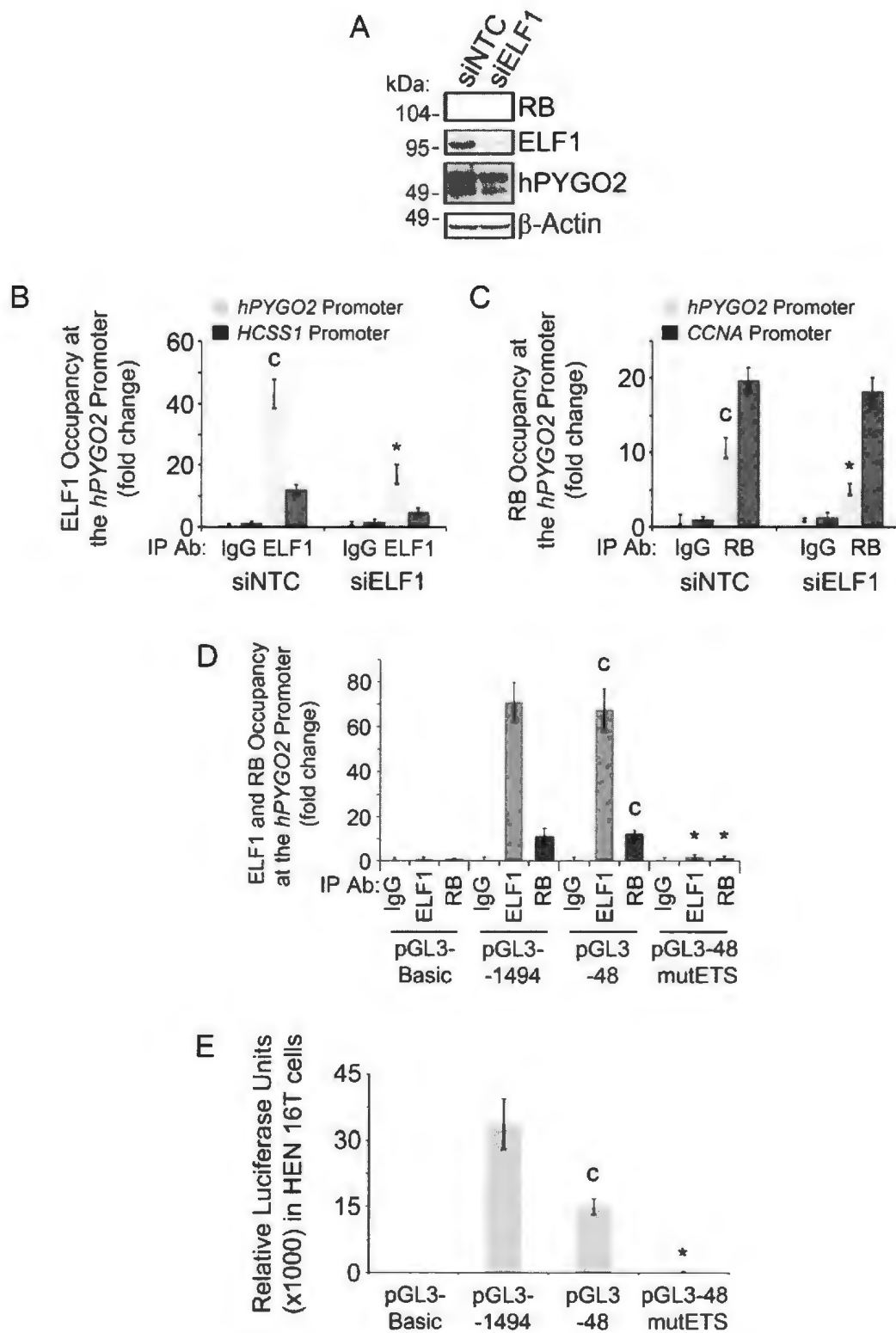
overexpressed in combination with ELF1 (11 fold above control, $p=0.024$). As expected, when PSM-RB was overexpressed with (RB-independent) DA-ELF1, its presence at the *hPYGO2* promoter was significantly reduced compared to when it was coexpressed with wildtype ELF1 (32% reduction, $p=0.015$). On the other hand, the presence of RB at the *CCNA* promoter increased binding whenever PSM-RB was overexpressed and was not affected by DA-ELF1. These results strongly suggested that RB associated specifically with chromatin at the *hPYGO2* promoter through its interaction with ELF1 (Figure 4.8 G).

I next assessed whether ELF1 was required for the presence of RB at the *hPYGO2* promoter. Treatment of HEN 16T cells with siELF1 effectively reduced ELF1 and *hPYGO2* protein levels but without affecting RB protein levels compared to the non-target control siRNA (Figure 4.9 A). Treatment with the siNTC control did not alter the presence of ELF1 and RB at the *hPYGO2* promoter and at the promoters of their target genes, *HCCS1* and *CCNA*, respectively (Figure 4.9 B and C). Treating cells with siELF1 significantly reduced ELF1 binding to both *hPYGO2* (2.5 fold, $p=0.024$) and *HCCS1* (2.4 fold, $p=0.031$) promoters. The presence of RB at the *hPYGO2* promoter was also significantly reduced (2.1 fold, $p=0.033$), but RB binding to *CCNA* was not affected. These results are consistent with aforementioned findings demonstrating specific ELF1-dependent association of RB with the *hPYGO2* gene promoter.

Given that the presence of the RB-ELF1 complex at the *hPYGO2* promoter depended on ELF1, I hypothesized that ELF1 may bind to one (or both) of the two putative EBSs in the promoter. I used site directed mutagenesis to change the sequence of

Figure 4.9 Requirement of an intact EBS and ELF1 presence for RB binding to the *hPYGO2* promoter in CxCa cells.

(A) HEN 16T cells were treated with either a non-targeting control (siNTC) or an ELF1 siRNA (siELF1). Immunoblots were performed to confirm the knockdown of ELF1 protein and levels of RB and hPYGO2 were determined. β -Actin was used as a loading control. (B-C) HEN 16T cells were processed for ChIP assays and immunoprecipitated with anti-ELF1 and anti-RB antibodies. A rabbit and mouse IgG mixture was used as a nonspecific control. Promoter regions of *hPYGO2*, *HCCS1* and *CCNA* were amplified by Q-PCR. HEN 16T cells were transiently transfected with pGL3-1494, pGL3-48 and pGL3-48 mutEBS luciferase constructs and a β -gal expression vector. pGL3-basic was used as an empty luciferase plasmid control. Cells were processed for ChIP assays (D) and immunoprecipitated with anti-ELF1 and anti-RB antibodies or a rabbit and mouse IgG mixture as a nonspecific control, or assayed for luciferase activity (E), which was normalized to β -gal activity. Bars represent standard deviation. Significant changes ($p < 0.05$) in expression, relative to the control/untreated sample (c), are indicated by an asterisk.



both putative EBSs in the pGL3-48 luciferase reporter plasmid thereby generating pGL3-48 mutEBS. ChIP assays were used to measure the association of RB and ELF1 to the *hPYGO2* promoter in each of the reporter plasmids (Figure 4.9 D). ELF1 promoter occupancy was 74.6 fold higher in pGL3-1494 and 70.9 fold higher in pGL3-48 relative to the empty plasmid control. RB binding to the *hPYGO2* promoters in pGL3-1494 (12.4 fold) and pGL3-48 (13.4 fold) was significantly higher than pGL3-basic. Mutating both EBSs completely abolished the association of RB and ELF1 to that of the level of the empty vector control.

To confirm that mutation the EBS in the *hPYGO2* promoter and subsequent loss of ELF1 binding resulted in loss of *hPYGO2* promoter activity, I performed luciferase assays with these reporter constructs. HEN 16T cells were transfected with pGL3-basic, pGL3-1494, pGL3-48 or pGL3-48 mutEBS and assayed for luciferase activity and RB and ELF1 promoter occupancy in the luciferase plasmids. Luciferase reporter activity levels in the pGL3-1494 and pGL3-48 samples were significantly higher (4.5×10^6 RLU, $p < 0.01$ and 2×10^6 RLU, $p < 0.01$) than in the empty vector control, pGL3-basic, (2.2×10^3 RLU) (Figure 4.9 D). The reporter activity level of the pGL3-48 mutEBS sample was slightly and non-significantly higher (979 RLU, $p = 0.061$) than pGL3-basic.

4.4 Discussion

The up regulation of *hPYGO2* in severely dysplastic lesions, cervical cancers and cervical cancer cell lines, along with its requirement for proliferation of these cells suggests that hPygopus2 plays an important role in CxCa pathogenesis. Activation of the Wnt signalling pathway and components of the pathway have been associated with cervical carcinoma (Uren et al., 2005). Thus, it is not unreasonable to suggest that the up regulation of *hPYGO2* in CxCa and in CIN which I report in this study, may be linked to, or be a prerequisite for Wnt signal transduction, given its role as a chromatin modifier (Mosimann et al., 2006). Our recent findings (Andrews et al, Accepted), however, have demonstrated an additional but strong requirement for malignant growth-associated ribosomal DNA transcription in HeLa cells, a cervical adenocarcinoma derived cell line. While these concurrent results do not necessarily preclude a role in Wnt signalling in cervical cancer, the association of hPygopus2 with cell cycle progression genes observed in the present study would also support its role in ribosomal DNA transcription.

My findings are consistent with others showing the overexpression and requirement of hPYGO2 in a broad range of cancers (Thompson et al., 2002; Andrews et al., 2007; Popadiuk et al., 2006; Wang et al., 2010; Chen et al., 2011; Brembeck et al., 2011; Watanabe et al., 2013), but they are novel because they demonstrated that the increase in hPYGO2 expression begins at a premalignant stage. Because not all high-grade (CIN III) lesions progress to cancer, specific and effective prognostic progression biomarkers are needed to predict when and if this progression will occur. This is particularly important because of the limitations with vaccines (low vaccination rates,

high costs, unavailability and only limited benefit in sexually active women) and screening techniques (lack of sensitivity or specificity) (Wang et al., 2008). The finding that hPygopus2 expression increases specifically in the CIN III precancerous stage indicates that hPYGO2 may be as effective biomarker to predict the transition from high-grade dysplasia to cancer. The increase in cytoplasmic hPYGO2 protein levels could be a result of the cells' inability to translocate these high levels of hPYGO2 to the nucleus at a fast enough pace. The increase in nuclear hPYGO2 suggest that a significant portion is being translocated. None the less, there is an obvious increase in hPYGO2 protein levels in CIN III.

High-grade lesions have an elevated probability for progressing to cancer because HPV genome integration results in the loss of the E2 regulatory region and subsequent E7 oncoprotein up regulation. Because the increase in hPygopus2 expression correlates with the HPV integration event, I hypothesized that the two were causally linked. By knocking down E7 expression I observed a decrease in hPYGO2 protein levels, which correlated with an increase in RB binding to the *hPYGO2* promoter while not affecting ELF1 levels or promoter binding. hPygopus2 levels were increased in cervical carcinoma because E7 up regulation caused the degradation of RB. Furthermore, the reduction of p53 by re-expression of hPygopus2 in cells depleted of ELF1, demonstrated a relationship between these two genes in deregulated growth during oncogenesis. This molecular interaction may be part of an important signalling axis for the identification and possible targeting of early stage disease.

ELF1 protein is continuously expressed and localizes to the cytoplasm, where it becomes activated through phosphorylation and glycosylation. These modifications cause its translocation to the nucleus, where it binds to target gene promoters and activates gene expression. My immunohistochemical analyses of dysplasia core samples revealed nuclear ELF1 in CIN III, suggesting that ELF1 is active, which is consistent with previous findings (Nicol et al., 2008). In the nucleus, RB interacts with the LxCxE motif of ELF1, inhibiting its transcriptional activity. I propose that following HPV integration, E7 displaces RB from ELF1 and results in ELF1-dependent *hPYGO2* induction.

Initially ELF1 was regarded as a lymphoid gene specific transcription factor. However, it is also regarded as an important activator or repressor of genes involved in several processes such as development (Janknecht et al., 1989; Jin et al., 2009; Calero-Nieto et al., 2010; Choi et al., 2011), mitogenesis (Moreau-Gachelin et al., 1988), oncogenesis (Moreau-Gachelin et al., 1988; Seth et al., 1989), and viral gene activation (Markovitz et al., 1992; Leiden et al., 1992; Clark et al., 1993). The bulk of research examining the role of ELF1 in cancer has focused on the expression of ELF1 in relation to other biomarkers, such as Vascular endothelial growth factor and PCNA, or with clinical correlates. Detailed studies of ELF1 gene targets in cancer include ELF1 activation of the TEK tyrosine kinase, endothelial gene, and binding to the *HCCS1* promoter. An efficient way to potentially identify all ELF1 gene targets in these cells would be to perform a genome wide chromatin association analysis and to functionally group these genes to identify in which processes ELF1 is involved.

Chapter 5: Summary

Our increased understanding of the molecular events that cause cancer, combined with advances in sensitive diagnostic technologies and more efficient therapies with reduced side effects has necessitated significant investment in the development of novel cancer biomarkers. Pygopus was identified as a Wnt/ β -Catenin transcriptional complex and has since been shown to play a more general role in chromatin remodeling (Carrera et al., 2008; Wright and Tjian, 2009; Andrews et al., 2009 and Chen et al., 2010). Human Pygopus2 (hPYGO2) was demonstrated to be overexpressed in several diverse types of tumours (including ovarian, breast, brain, colon and esophageal), and the cell lines derived from these tumours (Popadiuk et al., 2006; Andrews et al., 2007; Wang et al., 2010; Chen et al., 2011 and Brembeck et al., 2011; Watanabe et al., 2013; Moghbeli et al., 2013). High levels of hPYGO2 were required for tumour growth (Popadiuk et al., 2006; Watanabe et al., 2013) and for the proliferation of cancer cells in culture (Popadiuk et al., 2006; Andrews et al., 2007; Wang et al., 2010 and Chen et al., 2011), specifically permitting passage through the G1/S transition (Chen et al., 2011).

Despite its requirement for the proliferation of cancer cells and its tumour specific expression, little has been done to identify factors that promote its expression. Our lab previously demonstrated that the E74-like factor 1 (ELF1) is required for the induction of *hPYGO2* in breast cancer (BrCa) (Andrews et al., 2008). Another potential explanation for increased hPYGO2 expression is by gene duplication, as demonstrated by Zhang et al. in mandibulofacial dysostosis (Zhang et al., 2010). Based on these earlier findings, my research focused on characterizing the cell cycle dependent expression of hPygopus2 and identifying novel mechanisms by which it is regulated. Overall, my results suggested that

hPYGO2 is induced in the G1 phase of the cell cycle by factors (E_2 via $ER\alpha$ -SP1 and RB-ELF1) that promote cell cycle progression.

By collecting cells in each phase of the cell cycle I determined that hPygopus2 mRNA and protein were expressed in a cell cycle dependent manner; with the highest and lowest expression in G1 and G_0 , respectively. These results suggested that hPYGO2 expression might be used to distinguish cells in G1 phase from non-proliferating cells. The cell cycle dependent expression of hPYGO2 provided an explanation as to why its expression is not uniform in all cells of a tumour. Cells expressing hPYGO2 may be proliferating (ie. in G1, S, G2 or M phases), while cells lacking hPYGO2 expression may be non-proliferating (perhaps due to serum deprivation and/or hypoxia). Additionally, I found that the expression of hPYGO2 in the G2/M phases relative to its expression in the G1 phase was indirectly proportional to cell cycle length. These results suggested that the relative expression of hPYGO2 might be used to determine tumour cell proliferation rate.

In the remainder of this thesis I examined the mechanisms by which *hPYGO2* is regulated. In Chapter 3, I determined that in breast cancer (BrCa) cells, 17β -estradiol (E_2) enhanced hPygopus2 expression by inducing the formation of the Estrogen receptor alpha ($ER\alpha$)- SP1 transcription factor (SP1) complex at its promoter. I utilized $ER\alpha$ antagonists, RNAi and site-directed mutagenesis to show that $ER\alpha$ and SP1 require an intact estrogen response element half site and GC-box and functional DNA binding domains to occupy the *hPYGO2* promoter. Taken together, these results implied that in $ER\alpha$ + BrCa cells, $ER\alpha$ and SP1-mediated the E_2 induction of *hPYGO2*. Furthermore, I demonstrated that in $ER\alpha$ - BrCa cells, SP1 could still activate *hPYGO2* and other genes

required for cell proliferation. A potential implication of this work is the utilization of hPYGO2 expression to assist in chemotherapy selection for endocrine disrupter unresponsive BrCa tumours.

In the final Chapter I demonstrated that hPYGO2 overexpression in cervical dysplasia and cancer is due to the up regulation of the Human Papillomavirus (HPV) effector *E7*. HPV-E7 directed proteolysis of RB resulted in the release, and thereby activation, of ELF1 at the E26 transformation-specific binding site in the *hPYGO2* promoter. Furthermore, I found that ELF1-mediated stimulation of hPygopus2 expression was required for CxCa cell proliferation. These results supported the hypothesis that HPV integration, and as a result *E7* induction, leads to hPYGO2 overexpression in high grade cervical dysplasia and cancer via ELF1. A potential implication of this research is the utilization of hPYGO2 expression to predict which dysplasias will progress to invasive cancers.

The induction of *hPYGO2* by factors that promote cell cycle progression (ie. causative cancer agents) suggests that hPYGO2 expression is closely linked to the molecular mechanisms (E_2 via $ER\alpha$ and HPV integration via *E7* abrogation of RB) that result in cancer. Therefore, these results provide an explanation for the overexpression and requirement of hPygopus2 in tumours and support its future development as both a diagnostic and therapeutic cancer biomarker.

Future experiments to gain a better understanding of the role of hPygopus2 in cancer signalling networks and to further evaluate its utilization as a biomarker should begin with a survey examining hPygopus2 expression levels in an array of tumour types.

This analysis is supported because of its direct involvement with RB, which is rendered non-functional, and ELF1 and SP1, which are highly expressed, in multiple tumour types. Examination of coexpression, or lack thereof, between these factors and hPYGO2 on a tumour microarray containing several types of cancer would be a solid preliminary approach.

An insightful method to examine the requirement of hPYGO2 in various cancer types (initially in cell lines and eventually in mice) would be to use the FDA-approved drug Pyrvinium, which binds and inhibits Casein kinase I, but also promotes degradation of hPYGO2 (Thorne et al., 2010).

Although the function of hPygopus2 was not examined, the evidence regarding its expression, regulation and requirement, as presented within this thesis, suggests a role for hPYGO2 in the G1/S transition. This hypothesis is consistent with all of the previously ascribed functions of hPYGO2 including Wnt signalling, chromatin remodeling/transcription and ribosomal DNA transcription. As proliferating cells are only responsive to mitogens in the G1 phase, all of the above mentioned processes would be most active during this time. The induction and high expression of hPYGO2 during G1 would allow the unhindered execution and completion of the processes in which hPYGO2 is involved. This hypothesis is further supported by my results, and those of others, demonstrating the requirement of hPYGO2 for the transition from G1 into S. Elucidation of the exact role of hPYGO2 during G1 could be achieved by performing a chromatin immunoprecipitation-sequencing assay to determine which gene promoters hPYGO2 occupies during this phase.

Chapter 6: Appendix

Table 6.1 List of oligonucleotide sequences used in this thesis.

	Forward	Reverse
Gene Primers		
C-Actin	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG	ATG GCT GGG GTG TTG AAG GTC TC
ADA	GGC TTC GAC AAG CCC AAA GTA	CTC TGC TGT GTT AGC TGG GAG
CTSD	GGT TCC TCT GGA AGC CCT GTA G	TCC TGC ACC TGC TCG TCC
CCND	CCG TCC ATG CCG AAG ATC	ATG CTC AGC GGG AAG AC
EBAG9	AGT TCC TAA GCA GAC AGA TGT TG	CCC ATT CCC TCC TTC GAT CTT TA
ELF1	CCA GTC ACC CAT GTG TCC GTC AC	CAC AGA TAT ATT TGG CBT AGT GGC TGG GG
FOS	CCG GGG ATA GGC TCT CTT ACT	CCA GGT CCG TGC AGA AGT C
GAPDH	CCA TCA CAC CAC AGT TTC CC	CCA GAT CAT CAG CAA TGC C
HSPB1	ATC TGG AAA AGA ATA TCG CCG AC	TTG CBT GGC AGG TAT CTT GTT
IGFBP2	CAG GGG CAC TTG TGA GAA CCG	TGT TTA TGG TGC TGT CAA CBT G
PCNA	GAC CCG AAC CTG GCC AT	CGG TTA TCT TCG GCG CTT AGT
PRA	CTC TGC CTC GCA TGA AGA AG	CTT TGG TCA CGT CCA AGA CTT T
hPYGO2	GTC CCC CAC TCC ATG GGC CCG TCG	TGG CTT CTT TTC TGG ACT CTT C
RARA	AAG CTC GAG TCC TCT GAG A	TTT GTA GTG TAT TTG CCG AGC
RB	GAA CAT CGA ATC ATG GAA TCC CT	AGA GGA CAA GCA GAT TCA AAG TGA T
SP1	GGT GGC TTT TCA CAG GCT C	GCT GTT CTCA TTG GGT GAC TC
TER1	GGA GCA AGT TGG AAA GCA TTG	TCC CAC GAC GTA GTC CAT GTT
TGFA	AGG TCC GAA AAC ACT GTG AGT	AGC AAG CCG TTC TTC CCT TC
Promoter Primers		
CCNA	CCC CAG CCA GTT TGT TTC T	AGT TCA AGT ATC CCG CGA CT
CTSD	TCC AGA CAT CCT CTC TGG AA	GGA CCG GAG GGT CCA TTC
hHCS1	GAC ACC TGA CBT GAC AGG AA	GTC AGC CCG TCT GTC AUC
hPYGO2 (-1494 to -1143)	GGA AGC TTC GBT TTG GGT TGG GAT AAC AGA GG	GTC TAC TGC TTT ATA CTC TCA ATG C
hPYGO2 (-1143 to -829)	GCA GAT CTG CAT TGA GAC TAT AAA GCA GTA GAC	CTC AGT CTC CCG GTA AGA GAC C
hPYGO2 (-829 to -533)	GCA GAT CTG GTC TCT TAC CUC CAG ACT GAG	GGC GTA CAC AAT AAG CTT CCC AGA AG
hPYGO2 (-533 to -225)	GGA GAT CTC TTC TGG GAA GCG TAT TGT GTA CCG C	CTT GCG GGA CCG TAC CAT CC
hPYGO2 (-225 to +74)	GGA TGG TAC GBT CCC GCA AG	GCA AGC TTC CGA GCT CCA GCA ACC ACA AAG TG
hPYGO2 (-108 to +74)	GCA GAT CTC ACG CBT AGC GTG TGG TTC GGT C	GCA AGC TTC CGA GCT CCA GCA ACC ACA AAG TG
hPYGO2 exonic region primers are same as the hPYGO2 gene primers		
Site-Directed Mutagenesis Primer Sets		
RB Binding Site in ELF1 (Amino Acid Changes: LxCNE → R/Rut) (New Vector Name: pCS2+ELF1 → pCS2+DA-ELF1)		
	CAT GTC ATT GGG CTC ATG CAC TCT GCG ACG ACC	CAA TAG TTA TGC CCG TCG TGC CAG AGT GCA
	GGC ATA ACT ATT	TGA GCG CAA TGA CAT C
ETS in hPYGO2 Promoter (Nucleotide Changes: TTCCggTTCC → TCTAggTCTA) (New Vector Name: pGL3-48 → pGL3-48 mutETS)		
	GAT CTC GCG ACG AGT CTA GGT CTA GGT TGC TGC	CGG CCG CAG CAA CTT AGA CCT AGA CTC GTC
	CCG CG	CCG AGA TC
ERE Half-Site at -331 in hPYGO2 Promoter (Nucleotide Changes: TGCCC → TATAG) (New Vector Name: pGL3-531 → pGL3-531 mutERE)		
	GTG GGA GGC GAG CCT ATA GGG CCG CCC AGC AC	GTG CTG GGG CCG CTT ATA GGC TCG GCT CCC GC
GC-Box at -356 in hPYGO2 Promoter (Nucleotide Changes: CCGCCC → CTGTCC) (New Vector Name: pGL3-531 → pGL3-531 mutGC)		
	CGG GTG GGT CCA GAC AGG AGC CCG GGC	GGC CCG GCT CTT GTC TCG ACC CAC CCG
ERα DBD Mutant (Amino Acid Changes: CEGC → CAAC) (New Vector Name: pcERα → pcERα DBD)		
	GTG TGG TCC TGC GCA GCG TGG AAG GCG TTC	GAA GGC CTT GCA GGG TGC GCA GGA CCA GAC
SP1 DBD Mutant (Amino Acid Changes: KHKTHQ → KAKTAQ) (New Vector Name: pcSP1 → pcSP1 DBD)		
	CAC CTG TCA AAA CAT ATC AAG ACC CAC CAG AAT AAG	CTT ATT CTG GTG GGT CTT GAT ATG TTT TGA CAJ GTG
siRNA sequences		
siNC	GGA CCG UGG UCA ACC AUG UUU	
siPy2-X	GGA GAC AGC UUU AGG GAA UUU	
siPy2-Z	GGA GUU AGG UGA ACC AUG UUU	
siE#1	GAA AGA GAA CAC UGA GAA UUU	
siE7	CUA GUC UUU ACU GUU AUG AUC AAU U	
siSP1	AUC ACU CCA UGG AUG AAA UGA UU	

Table 6.2 List of antibodies and conditions used in this thesis.

Primary Antibodies	Species	Dilution	Company/References	Clone No.
β -Actin	Mouse	1:4000	Sigma	AC-15
CCND	Mouse	1:1000	BD Pharmingen	556470
CTSD	Mouse	1:500	Dako	A0561
ELF1	Rabbit	1:4000	Santa Cruz Biotechnologies	C-20X
ER α	Rabbit	1:5000	Santa Cruz Biotechnologies	HC-20
HPV16 E7	Mouse	1:1000	Abcam	Ab20191
HPV16 L1	Mouse	1:250	Abcam	Ab2417
IgG	Rabbit		Santa Cruz Biotechnologies	sc-2027
IgG	Mouse		Santa Cruz Biotechnologies	sc-2025
Ki67	Part of Ventana Kit			
p16INK4	Part of Ventana Kit			
p21	Mouse	1:1000	Santa Cruz Biotechnologies	C-19
p53	Mouse	1:1000	Santa Cruz Biotechnologies	DO-1
PCNA	Mouse	1:1000	Sigma	F-2
phospho-Histone H3	Rabbit	1:1000	Cell Signaling	S10, D2C8
PYGO2	Rabbit	1:1000	Generated in house	Haussen
PYGO2	Rabbit	1:1000	Generated in house	Scratchy
RB	Mouse	1:1000	BD Pharmingen	554136
SP1	Mouse	1:1000	Santa Cruz Biotechnologies	sc-420
SecondaryAntibodies	Species	Dilution	Company/References	Clone No.
Sheep anti-mouse horseradish peroxidase		1:2000	GE Healthcare	NA934V
Donkey anti-rabbit horseradish peroxidase		1:4000	GE Healthcare	NA931V
Biotin Goat anti-rabbit IgG		1:200	Zymed	62-6140
Biotin Goat anti-mouse IgG		1:200	Zymed	62-6540
Horseradish peroxidase streptavidin		1:200	GE Healthcare	RPN1231N
Donkey anti-rabbit fluorescein isothiocyanate		1:250	Jackson ImmunoResearch Laboratories	711-095-152
Donkey anti-mouse Cy3		1:250	Jackson ImmunoResearch Laboratories	715-165-150

Figure 6.1. Cell cycle distribution plots for the phase representative samples of six cell lines.

Samples from the six cell lines were collected as they progressed through the cell cycle after SD and release. Distribution plots of the representative samples for each cell cycle phase and the percentage of cells in the appropriate phases are shown

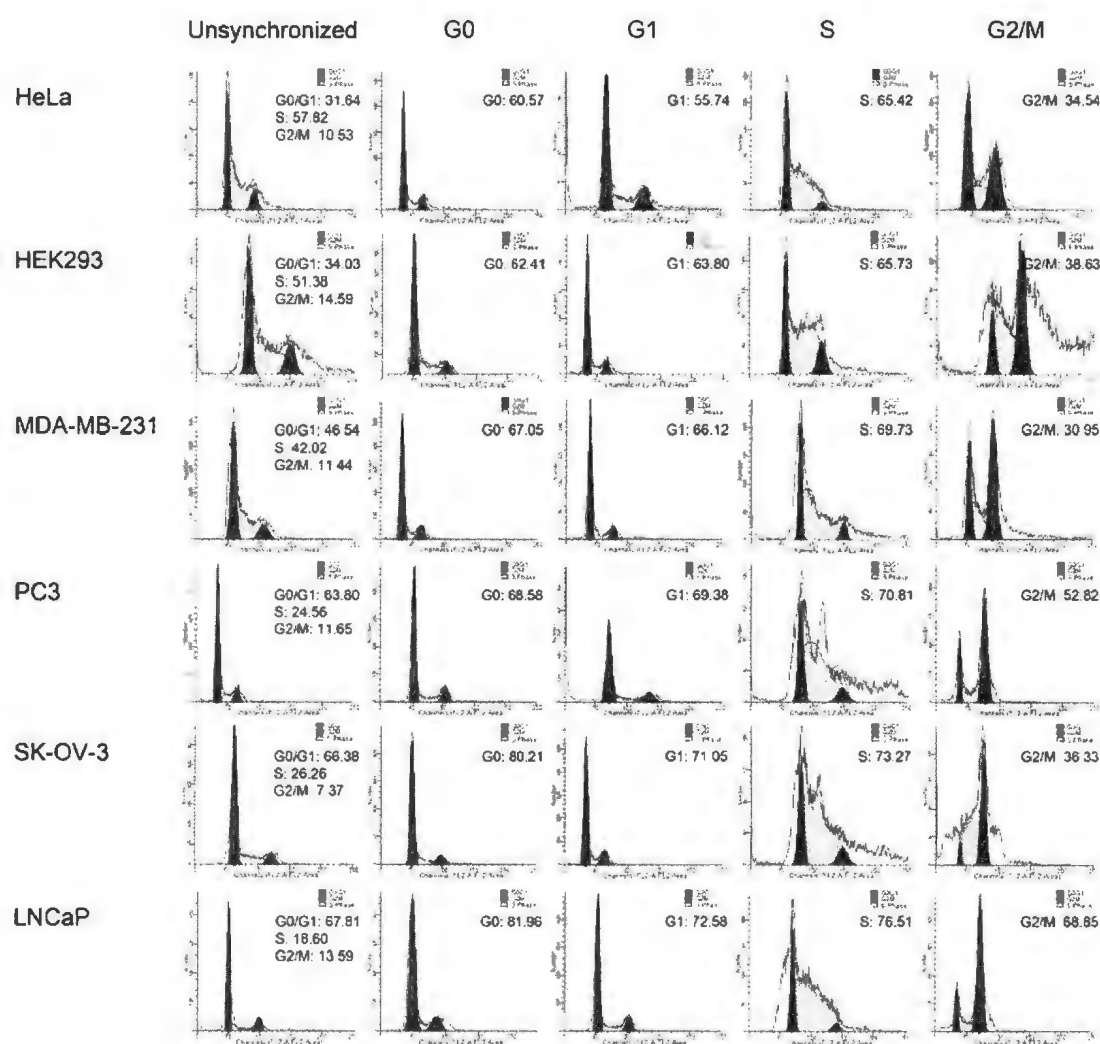


Figure 6.2 E₂ induction of genes in VC5 and MC2 BrCa cell lines

Comparison of E₂ induced gene expression between the ER α - parental MDA-MD-231 cell lines, the ER α constitutively expressing stable transfectant cell line (MC2) and the empty vector control cell line (VC5). Genes examined were selected according to a review article by the group that generated these cell lines (Levenson and Jordan, 2004). E₂ induction of Progesterone receptor (PGR), Tumor growth factor alpha (TGF α), Insulin-like growth factor binding protein 4 (IGFBP4), Retinoic acid receptor alpha (RAR α), Cathepsin D (CATD) and v-myc myelocytomatosis viral oncogene homology (MYC) is consistent with the levels in the review.

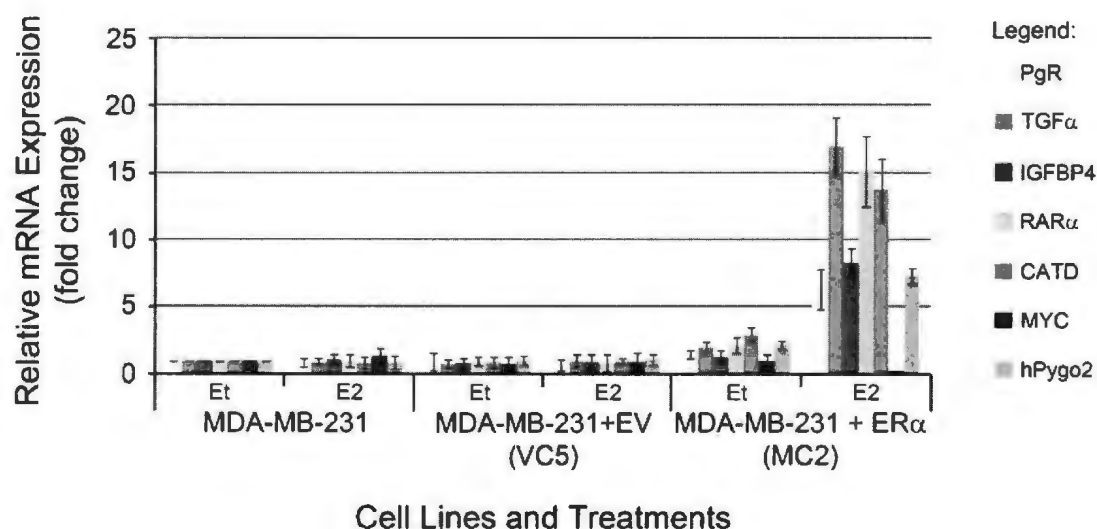


Figure 6.3 ER α translocation to cytoplasm from nucleus after treatment with FUL

Constitutively expressing ER α MC2 cells were treated with ethanol (Et), 17 β -estradiol (E₂) or fulvestrant (FUL) and protein levels of ER α , β -Actin and β -Tubulin were measured by immunoblot in either whole cells (WC) or extracted nuclei (N).

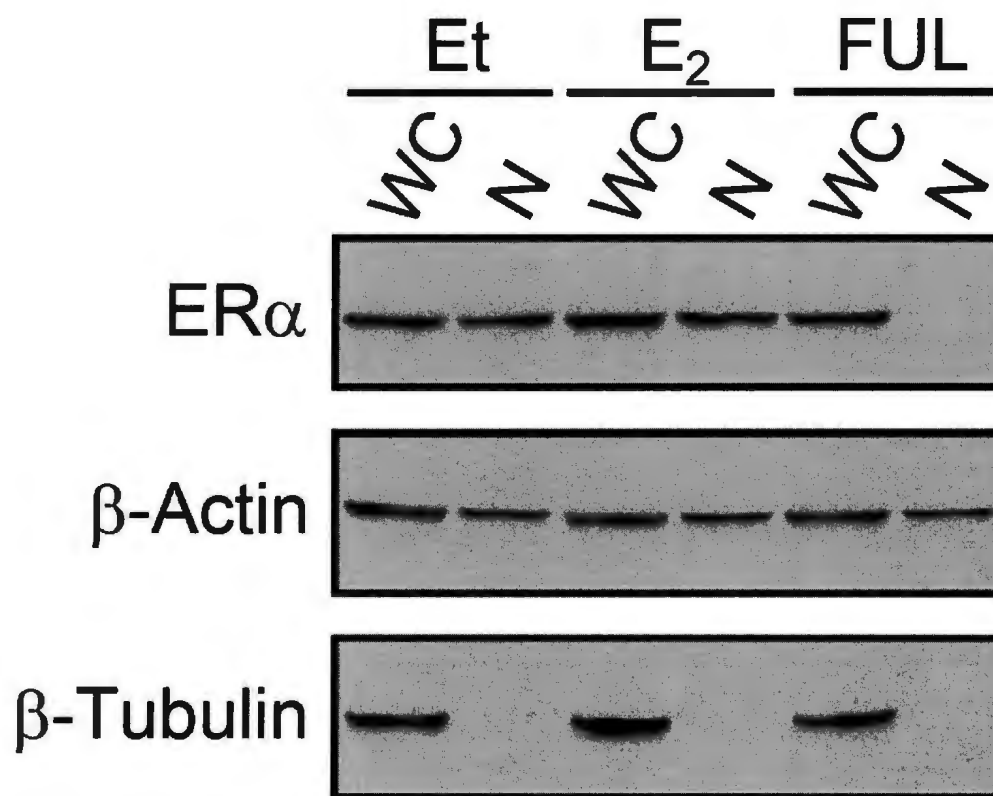


Figure 6.4 Statistical analyses for hPYGO2, p16INK4a and MKI67 staining on a CIN microarray.

An Ordinal Logistic Random Effects Model was fit to the data. The response was taken to be the stain intensity, (a 0 value corresponding to Negative Staining, a 1 corresponding to Weak Staining, a 2 corresponding to Strong Staining). Further, the covariates in the model were the Diagnoses (increasing in severity from Normal to CIN1, to CIN2, to CIN3, to SCC), the observer doing the counting (KK, PA, and YRT), the antigen used to stain (hPYGO2, MKI67, and p16) as well as a random effect for the Core Sample used (18 values). An ANOVA confirmed that the random effect of Core Sample was significant ($p < 10^{-16}$). Moreover, the model was constructed with "difference contrasts" on Diagnoses. That is, our contrast matrix has the form:

Normal	0	0	0	0
CIN 1	1	0	0	0
CIN 2	1	1	0	0
CIN 3	1	1	1	0
SCC	1	1	1	1

The interpretation of the model coefficients for this contrast matrix is given in the Table. All estimates are relative to the baseline of Observer KK, Antigen hPYGO2, and Normal Disease. We see that only when we stain samples with Diagnoses of CIN3 or SCC that we have a significant increase in stain intensity ($p < 0.05$). We note that although it appears that SCC stains less intensely than CIN3 the difference is not significant ($p = 0.12$). That is, Pygopus stains more intensely for advanced disease states.

Cumulative Link Mixed Model fitted with the Laplace approximation

What is significant to note is the all coefficient estimates for the incremental increase in disease status are positive (and significant), implying that stain intensity with Pygopus increases with Disease Severity.

TABLE: p values for staining differences observed for Pygopus with disease progression

Random effects: Core.ID Var: 0.6859166 Std.Dev: 0.8282008

	Estimate	Std. Error	z value	Pr(> z)
Observer PA	-0.4433	0.0382	-11.6174	$< 2.22 \times 10^{-16}$
Observer YT	-0.5053	0.0282	-17.8911	$< 2.22 \times 10^{-16}$
change in going from N to CIN1	0.3655	0.7098	0.5149	0.6065977
change in going from CIN1 to CIN2	0.4883	0.6339	0.7703	0.4411318
change in going from CIN2 to CIN3	1.2559	0.5874	2.1379	0.0325259
change in going from CIN3 to SCC	0.9166	0.5877	1.5596	0.1188545
Difference from antigen MKI67	-1.2371	0.0348	-35.5348	$< 2.22 \times 10^{-16}$
Difference from antigen p16	-0.4359	0.0315	-13.8379	$< 2.22 \times 10^{-16}$

No scale coefficients

Threshold coefficients:

Estimate Std. Error z value

0|1 0.0087 0.5758 0.0151

1|2 1.8073 0.5759 3.1381

log-likelihood: -25042.57

AIC: 50107.14

Table 6.3 Generation and characterization of HEN, HEC, HEN 16T and HEC 18T cell lines.

Generation				
Isolation	HEN cultures were derived from a cervical specimen obtained from hysterectomy (donor age, 44 years)			
	HEC cultures were derived from a cervical specimen obtained from hysterectomy (donor age, 36 years)			
Culturing	Serum free DMEM + BPE +EGF Serum free DMEM + BPE			
Generation of HEN 16T cells	HEN cells transfected with HPV16 (integrated) and repeatedly treatments with cigarette smoke condensate (CSC)			
Generation of HEC 18T cells	HEC cells were transfected with HPV18 (integrated) and repeatedly treated with CSC			
Culturing	HEN 16T and HEC 18T were grown in DMEM + 10% FBS			
General Characteristics				
	HEN	HEN 16T	HEC	HEC 18T
Immortalized	No	Yes	No	Yes
HPV DNA	Absent	Integrated	Absent	Integrated
Ploidy	Diploid	Aneuploid	Diploid	Aneuploid
In vivo Proliferation Characteristics				
Nodule formation	No	Yes	No	Yes
Tumour formation	No	Yes	No	Yes
Epithelium Structure	Thin but multilayered	Thick, aberrant and disorganized, loss of cell arrangement	Differentiated stratified squamous epithelium	Dysplastic cells throughout, loss of differentiation
nucleus	Regular	Enlarged and hyperchromatic	N/A	N/A
invasion	No	Yes	No	Yes
Reminiscent to	Mature metaplasia	Severe dysplasia	Squamous epithelium	Severe dysplasia
Gene Expression (mRNA levels/protein levels)				
E6	-	+++	-	+++
E7	-	+++	-	+++
TERT	-	++	-	++
HRAS	+	++	N/A	
CMYC	+	++		
BMYB	-	++		
TP53	-/++	++/+		
CDKN1A (p21)	++	++		
CDKN2A (p16)	-/-	++/++		
PCNA	-/-	++/++		
BAK1	+	++		
BAX	+	+		
BCL2	+	+++		
BCL2L1	+	+++		
BAG1	+	++++		

Figure 6.5 Presence of HPV 16 and 18 DNA and *E6* and *E7* mRNA expression in CxCa cell lines.

(A) HPV 16 and 18 oligonucleotides were used to amplify HPV DNA in two normal cervical cell lines (HEN and HEC) and six CxCa cell lines (C33a, HeLa, CaSki, SiHa, HEN 16T and HEC 18T) by reverse transcription PCR. Levels were normalized to the NHD region of *hPYGO2*. (B) Oligonucleotides pairs were used to amplify HPV 16 *E6* and *E7* and HPV 18 *E6* and *E7* genes in the normal and CxCa cell lines. mRNA levels were normalized to β -Actin.

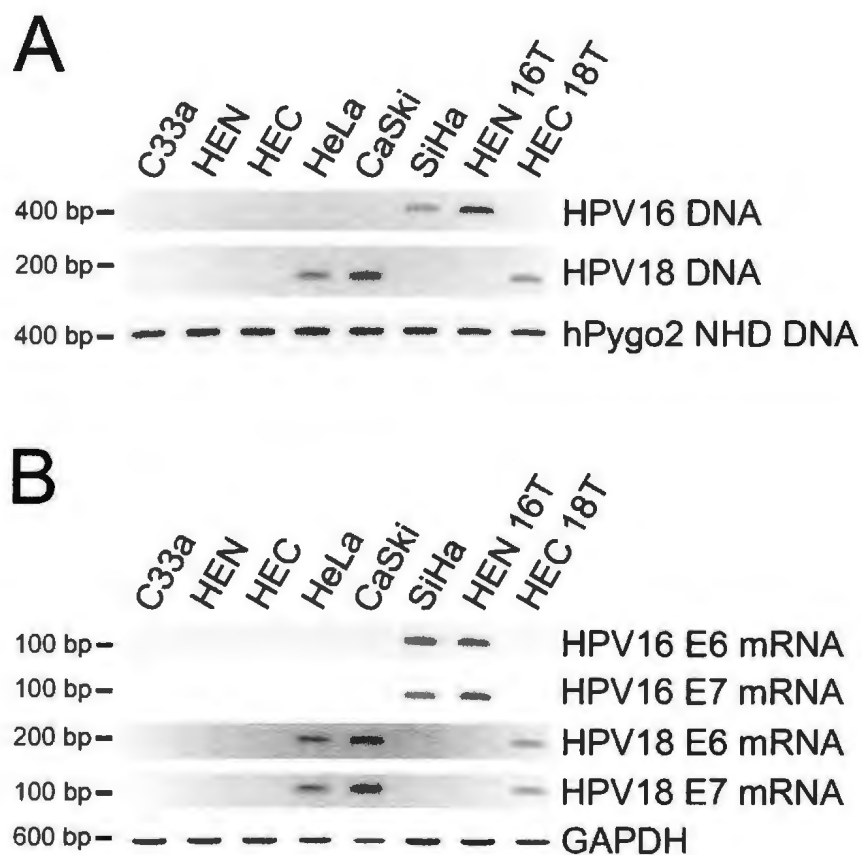
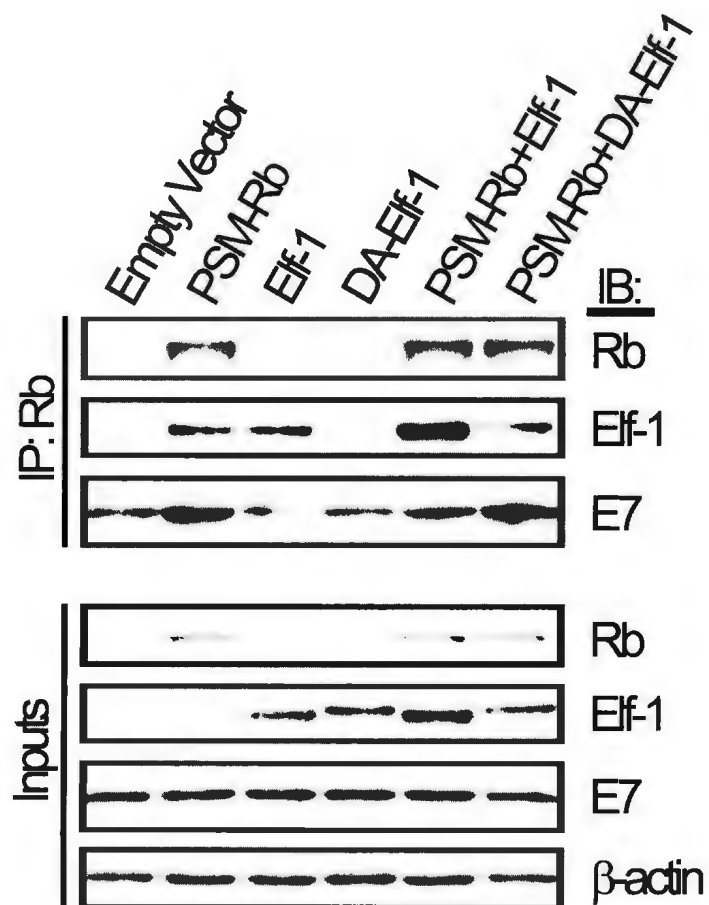


Figure 6.6 Characterization of RB and ELF1 binding characteristics in HEN 16T cells.

Immunoprecipitations demonstrating the binding dynamics between phosphorylation site mutant (RB PSM-RB) and wildtype ELF1, dominant active ELF-1 (DA-ELF1) and HPV 16 E7.



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